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Program: Immunology

Methods for *Leishmania* parasite detection and quantification as a tool for study of the pathogen-vector-host interactions

Thesis for the degree of Philosophiae Doctor (PhD)

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Declaration:

I confirm that this thesis was worked up by me. All literature and other sources of information used for preparation of the thesis were cited. The present work or any significant part of it has never been used for acquirement of the same or similar degree.

Prohlášení:

Prohlašuji, že jsem disertační práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Leishmaniasis in human is caused by total 21 species of the intracellular protozoan parasite *Leishmania*, which are transmitted by about 30 species of phlebotomine sand flies. Besides human, *Leishmania* can infect a number of vertebrate hosts. The major host cell is the macrophage, in which parasites multiply, eventually rupturing the cell and spreading to uninfected cells. Infected monocytes and macrophages circulating in the peripheral blood are thought to be carriers of the parasite to distal sites. Depending on the infected sites of the body, there are three forms of leishmaniasis: cutaneous, mucocutaneous and visceral. Leishmaniasis is a disease for which we still lack effective, affordable and easy to use drugs. In addition, surveillance and control are also neglected.

This thesis summarizes the results of several projects using different approaches for parasite load measurement in the mouse model of leishmaniasis, including two methods that were developed and optimized in our laboratory. Detection and quantification of pathogens belongs to the major topics of the research of various infectious diseases. This parameter is necessary for confirmation of the diagnosis, characterization of the host defense, complex pathological changes in the infected organisms, and for the evaluation of the effectiveness of therapy. We use quantification of *Leishmania* parasites to study influence of the genotype on susceptibility to the disease and to estimate the effectiveness of immunization with sand fly saliva.

Application of various parasite measurement assays revealed gene- and sex-dependent differences in susceptibility to *Leishmania.* Two genetic loci that control parasite dissemination in the internal organs were first detected by our laboratory in genome-wide screening using a mouse model of leishmaniasis. The obtained knowledge brings more complex understanding of the pathogen-vector-host interactions.

Metody detekce a kvantifikace parazitů rodu *Leishmania* jako nástroj pro studium vzájemného působení patogenu, vektoru a hostitele

Leishmaniáza je u lidí působena 21 druhem vnitrobuněčného jednobuněčného parazita rodu *Leishmania*, který je přenášen přibližně 30 druhy flebotomů. Kromě člověka jsou leishmánie schopné infikovat celou řadu obratlovců. Hlavní hostitelskou buňkou, v níž se parazit množí, je makrofág; posléze leishmánie buňku ničí a infikují další buňky. Nakažené monocyty a makrofágy cirkulují v periferní krvi a roznášejí parazita do dalších orgánů a tkání. V závislosti na infikované část těla jsou rozlišovány tři typy leishmaniázy: kožní, kožně-slizniční a viscerální. Pro léčbu leishmaniázy stále ještě chybí účinné, dostupné a jednoduše aplikovatelné léky. Zanedbaná je také diagnostika a monitorování onemocnění.

Tato disertační práce shrnuje výsledky několika projektů, ve kterých byly použity různé přístupy k určení množství parazitů v myším modelu leishmaniázy, včetně dvou metod, které byly vyvinuty a optimizovány v naší laboratoři. Detekce a kvantifikace patogenů patří k hlavním tématům výzkumu různých infekčních chorob. Tento parametr je nezbytný pro potvrzení diagnózy, charakterizaci imunitní odpovědi hostitele a komplexních patologických změn v nakaženém organismu, a také pro posouzení účinnosti léčby.

Kvantifikaci leishmanií jsme použili pro studium vlivu genotypu na vnímavost k onemocnění a pro určení efektivity imunizace hostitele slinami flebotomů. Využití různých metod měření množství parazitů vedlo k nalezení rozdílů ve vnímavosti k leishmaniím, které závisely na genotypu a na pohlaví. Citlivost použité metody vedla k prvnímu zmapování dvou genů, které kontrolují rozšíření parazitů do vnitřních orgánů, v celogenomovém skríningu. Získané znalosti přinášejí lepší porozumění interakce mezi patogenem, vektorem a hostitelem.

Content

List of abbreviations

ABTS	2,2′- azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid)
ANOVA	analysis of variance
asat	aspartate aminotransferase gene
BSA	bovine serum albumine
CCL2/MCP-1	chemokine ligand 2 / monocyte chemotactic protein-1
CCL3/MIP-1α	chemokine ligand 3 / macrophage inflammatory protein-1α
CCL4/MIP-1β	chemokine ligand 4 / macrophage inflammatory protein-1β
CCL5/RANTES	chemokine ligand 5 / regulated upon activation, normal T-cell expressed, and secreted
CCL7/MCP-3	chemokine ligand 7 / monocyte chemotactic protein-3
CD4	cluster of differentiation 4
CL	cutaneous leishmaniasis
cpb	cysteine protease b gene
cyt b	cytochrome b gene
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
DAT	direct agglutination test
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
fh	fumarate hydratase gene
g6pdh	glucose-6-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
gp63	63 kDa glycoprotein gene

gpi	glucose-6-phosphate isomerase gene
GTPase	guanosine triphosphate hydrolase
H&E	hematoxylin and eosine stain
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
hsp70	70 kDa heat shock protein gene
IDO	indoleamine-2,3-dioxygenase
IFNγ	interferon gamma
icd	isocitrate dehydrogenase gene
Ig	immunoglobulin
iNOS	inducible nitric oxide synthase
IL	interleukin
ITS	internal transcribed spacer
kDa	kilodalton
kDNA	kinetoplast deoxyribonucleic acid
L.	*Leishmania* genus
LAT	latex agglutination test
Leishmania spp.	a group of strains that belong to *Leishmania* genus
Lmr	*Leishmania major* response locus
LST	leishmanin skin test
MCM	microcapillary culture method
me	malic enzyme gene
ML	mucocutaneous leishmaniasis
MLEE	multilocus enzyme electrophoresis
MON system	Montpellier system for *Leishmania* species identification
mpe	mannose phosphate isomerase gene

NADH	nicotinamide adenine dinucleotide
NO	nitric oxide
nh1	nucleoside hydrolase 1 gene
nh2	nucleoside hydrolase 2 gene
P.	*Phlebotomus* genus
PBS	phosphate buffer saline
PCG	promastigote secretory gel
PCR	polymerase chain reaction
pgd	6-phosphogluconate dehydrogenase gene
PS-PCR	polymorphism-specific polymerase chain reaction
QTL	quantitative trait locus
RCS	recombinant congenic strain
RC strains	recombinant congenic strains
RDB	reverse dot blot
RI strains	recombinant inbred strains
RLB	reverse line blot
RFLP	restriction fragment length polymorphism
rK39	recombinant K39
RPMI	Roswell Park Memorial Institute medium
rRNA	ribosomal ribonucleic acid
RT-PCR	real-time polymerase chain reaction
s.c.	subcutaneously
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNB-9	saline-neopeptone-blood-9 medium
SNP	single nucleotide polymorphism
ssu-rRNA	ribonucleic acid of the small ribosomal subunit

SYBR-green	cyanine dye; N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
Th	T helper cell
TNF	tumor necrosis factor
TRITC	isothiocyanate derivative of rhodamine
VL	visceral leishmaniasis

Introduction

I. Leishmaniasis in the world

Various intracellular pathogens, which cause a wide spectrum of diseases, live and replicate within endosomal compartments or the cytosol of diverse host cells. Examples of the intracellular infectious agents include all viruses, many different bacteria (such as *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Brucella spp*., *Salmonella spp*.) as well as certain protozoa (*Leishmania spp*., *Toxoplasma gondii*, *Trypanosoma cruzi*) and fungi (such as *Histoplasma capsulatum*) (Bogdan 2008).

Leishmaniasis is a vector-borne protozoan infection with a wide clinical spectrum. About 21 *Leishmania* species are known to infect human, they are transmitted by approximately 30 species of flebotomine sand flies (Herwaldt et al. 1999, Pavli et al. 2010). *Leishmania* amastigotes have been first observed by Cunningham (1885) in human skin lesions of patients from India, but he suggested that they were members of Mycetozoa (fungi) (Cunningham 1885). Protozoal nature of *Leishmania* was first recognized by Borovsky (1898) during his study of skin lesions in Turkmenistan (Borovsky 1898). Leishmnan (1903) discovered similar intracellular bodies in the visceral organs of fatal cases of kala-azar from India, and established that they were morphologically related to trypanosomes (Leishman 1903). Similar observation has been made in the same year in India by Donovan (Donovan 1903).

There are extensive discussions about origins and evolution of *Leishmania* genus (Momen et al. 2000, Tuon et al. 2008), though it is proved that people suffered from this disease at least 4 000 years ago. DNA of *L. donovani* was found in ancient Egyptian and Christian Nubian mummies (Zink et al. 2006). The presens of *Leishmania* was also detected in the facial lesions on ancient skulls from the Atacama Desert in Chile (Costa et al. 2009).

1. Host-parasite interactions

At the different stages of development, *Leishmania* parasitize in two organisms: a flebotomine sand fly vector and a mammalian host (Figure 1, Lang et al. 2009). Parasites reside in neutrophils, monocytes and macrophages, as well as in dendritic cells and fibroblasts. *Leishmania* have a great potential for damaging bodily functions (Lipoldová and Demant 2006).

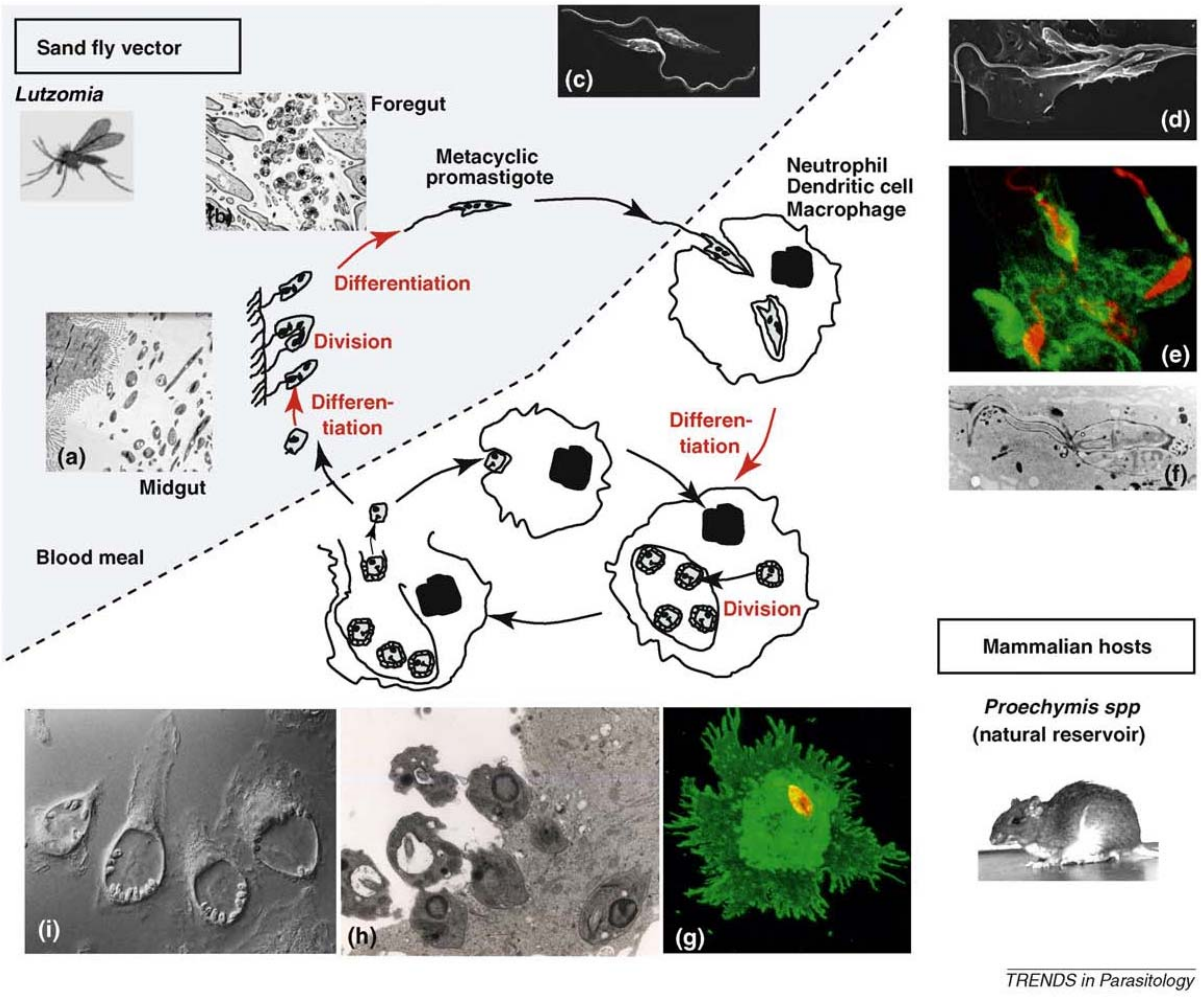


Figure 1. Life cycle of *Leishmania*

Leishmania are dimorphic parasites, existing as flagellated promastigotes in the digestive tract of the sand fly and as intracellular amastigotes within the cells of the mammalian host. When a sand fly feeds on a parasitized tissue, amastigotes are ingested with the bloodmeal and pass directly in the abdominal part of the midgut. Within 24 h, amastigotes

transform into promastigotes. These forms multiply rapidly, migrate from the anterior part of the midgut (Figure 1a) to the foregut (Figure 1b) and differentiate into metacyclic promastigotes (Figure 1c) ready to be inoculated during the next bloodmeal. After transmission of infectious promastigotes into the dermis of the mammalian host, parasites are phagocytosed by resident or recruited cells of the monocyte lineage including macrophages (Figure 1d-i) and dendritic cells (Figure 1g). Granulocytes are also well-suited target cells. Within minutes after phagocytosis, promastigotes are located into phagosomal compartment (Figure 1e-f). These phagosomes then undergo remodelling via the fusion with endocytic organelles and form a parasitophorous vacuole (Figure 1f-i). Within the vacuole, promastigotes transform and replicate into non-motile amastigotes which colonize additional cells (Lang et al. 2009).

The control of intacellular bacteria and protozoa, including *Leishmania*, usually requires $CD4^+$ T cells and IFNγ and/or TNF-dependent activation of macrophages. This leads to upregulation of antimicrobial effector mechanisms, including the acidification of the phagosomes and the expression of inducible nitric oxide synthase (iNOS), indoleamine-2,3-dioxygenase (IDO) and interferon-inducible GTPase. Antibody-mediated regulation of infections with intracellular pathogens might be due to Fc-receptor-mediated faciliation of the entry into the host cell of macrophage deactivation by inhibitory Fc receptors. In a case of leishmaniasis, the key effector pathways of macrophage against the intracellular parasite stage during acute phase of infection include IFNγ and iNOS, which converts arginine into citruline and leishmanicidal NO. However, it is important to note that the expression of iNOS alone is not necessary sufficient to control *Leishmania*. Even after clinical resolution of skin lesions, small quantities of parasites may persist in the dermis and draining lymph nodes (Bogdan et al. 2008). Studies of mouse models of leishmaniasis have provided important insights into response of the host to the infection. Several initial studies indicated that resistance or susceptibility to infection that is caused by *L. major* might be determined by the activation of

different classes of T helper cells, because disease progression seemed to be associated with a more prevalent response by T helper 2 (Th2) lymphocytes, which produce interleukin-4 (IL-4), whereas disease resolution seemed to correlate with a higher level of activation of Th1 cells that produce interferon-γ (IFNγ). However, subsequent studies indicated that additional immunological factors are also involved in the host response. IL-10 production by regulatory T cells has been suggested to explain a non-cure response in some mice that exhibit a Th1 response; IL-12 that is released by macrophages is an important factor in the stimulation of the Th1 response to leishmaniasis. Dendritic cells and neutrophils also have regulatory roles in stimulating either Th1 or Th2 responses to infection. A range of studies revealed a complexity of responses in relation to susceptibility or resistance to leishmaniasis, which are not easily integrated into a simple functional model and are influenced by multiple genetic factors (Lipoldová and Demant 2006).

2. Clinical forms and epidemiology of leishmaniasis

Leishmaniasis includes asymptomatic infection and three main clinical syndromes (Figure 2). In the dermis, parasites cause the cutaneous form of the disease (CL), which can be localized or diffuse; in the mucosa, they cause mucocutaneous leishmaniasis (ML), and the metastatic spread of infection to the spleen and liver leads to visceral leishmaniasis (VL; also known as "kala-azar"). Parasites can also enter other organs, such as lymph nodes, bone marrow and lungs, and in rare cases, can even reach the brain.

Figure 2. Clinical forms of leishmaniasis

Leishmaniasis is one of the most neglected diseases in developing countries, along with other like sleeping sickness or Chagas' disease. Such diseases are named "neglected" because they persist predominantly in marginalized and poor communities (Lipoldová et al. 2006, Dujardin et al. 2008, Machado et al. 2009, Pavli et al. 2010). The type of pathology that is caused depends on the species of *Leishmania*, the genotype and nutritional status of the host, the transmitting vector and environmental and social factors (Lipoldová and Demant 2006). The disease remains a public health problem worldwide, affecting approximately 12 million people in 88 countries; 50 000 die of this disease each year. Leishmnaniasis is endemic in areas of tropics, subtropics, and southern Europe, in setting ranging from rain forests in the Americas to desert western Asia. Leishmaniasis represents a major public health problem in the Eastern Mediterranean Region. Based on geographical distribution, the disease is divided into Old World and New World leishmaniasis (Herwaldt et al. 1999, Dujardin et al. 2008, Pavli et al. 2010, Postigo 2010).

Visceral leishmniasis is endemic in more than 60 countries in tropical and subtropical areas, and in Mediterranean countries, however 90% of the 500 000 new cases that occur every year concern six countries only – India, Bangladesh, Nepal, Brazil, Ethiopia and Sudan. VL is caused mainly by *L. donovani* in the Indian subcontinent, Asia and Africa; *L. infantum* in the Mediterranean basin, and *L. chagasi* in South America. In Mediterranean countries and South America, the disease is zoonotic and affects mainly infants and young children. In these countries, stray and domestic dogs are the main reservoir for the infection. In the Indian subcontinent and Africa, VL is anthroponotic and affects adults and children (Pavli et al. 2010).

Cutaneous leishmaniasis is endemic in more than 70 countries, with an estimation of 1.5-2 million new cases every year. Afghanistan, Syria, and Brazil are the main foci. CL caused mainly by *L. tropica* and *L. major* in the Old World, and by *L. braziliensis*, *L. guyanensis*, *L. panamensis*, *L. peruviana*, *L. mexicana*, *L. amazonensis*, and *L. venesuelensis*

in the New World. Mucosal leishmaniasis develops in a small number of patients with New World CL, however its course is chronic and may be life-threatening (Pavli et al. 2010).

The disease is spreading because of several risk factors. Climate changes, population movements, long-distance tourism and trade could play major roles in transcontinental transport of microorganisms (Dujardin et al. 2008). In the last decade, leishmaniasis expanded or emerged in several foci worldwide as the result of sand fly expansion due to natural and human factors, such as urbanization and deforestation, and global warming (Pavli et al. 2010).

International travel is growing rapidly worldwide. More than half people travel for leisure, though an increasing number of travelers are immigrants visiting friends and relatives in tropical and subtropical areas. In developed, non-endemic countries, there is and increasing number of cases of imported leishmaniasis in association with increasing international tourism, military operations, the influx of immigrants from endemic areas, and HIV-infected persons. Cases of leishmaniasis were described in organ transplant recipients. Cutaneous leishmaniasis is one of the top 10 diseases among tourists returning from tropical countries with skin problems (Machado et al. 2009, Pavli et al. 2010).

3. Leishmaniasis in Europe

The risk for reintroduction of some exotic vector-borne diseases in Europe has become a hot topic. Leishmaniasis is endemic in all southern European countries (Portugal, Spain, France, Italy, Greece, Cyprus, Turkey), with approximately 700 new cases per year; 3 950 if Turkey is included (Dujardin et al. 2008). In the European Union, the main threat comes from the spread of two parasites, *L. infantum*, which cause zoonotic visceral and cutaneous leishmaniasis in humans and domestic dogs, and *L. tropica*, which causes anthroponotic cutaneous leishmaniasis. Infection with *L. donovani* has also been reported. Increasing dog travel poses a significant risk of introducing *L. infantum* into northern Europe. Risks of emergence leishmaniasis in Europe are specifically associated with traveling, the natural

spread from Mediterranean region to neighboring temperate areas where there are vectors yet without disease, and increased number of immunosuppressed people (Dujardin et al. 2008, Ready 2010).

Complex diagnostics of leishmaniasis is based on criteria that include epidemiological data, clinical manifestations and laboratory tests. The lack of universal standardized techniques and experienced medical personnel can become a severe problem, especially in those countries where leishmaniasis is relatively rare. One of striking examples of the complicated case in Europe is delayed diagnosis of visceral leishmaniasis in 15 month old German child (Bogdan et al. 2001).

II. Mouse models used for genetic studies

Development of the complex diseases is controlled by multiple genes. Balance between the effects that contribute to resistance and susceptibility results in different clinical development of the pathological state in genetically diverse individuals. Scientists that perform human studies of complex diseases meet significant complications due to the genetic heterogeneity of outbred human populations, extensive gene interactions, variations in allelic frequencies and the incomplete penetrance of disease-causing alleles (Weiss and Terwilliger 2000). Moreover, development of the pathological condition is highly influenced by the wide range of social and environmental factors (nutrition, hygienic conditions, medical care, climate etc.) that are hardly to be standardized.

Animal models help to overcome problems of human studies and understand the genetic basis of polygenic diseases. Sequencing of the mouse genome and detailed analysis of homology between human and murine genes (Nadeau et al. 1990, Waterson et al. 2002, Brown et al. 2006) made possible to use a mouse model as a reliable tool to identify chromosomal loci that influence many diseases, including leishmaniasis. Analysis of polymorphism in the mouse genome revealed a mosaic structure with long segments of either

extremely high or extremely low polymorphism rates. Only one-third of the mouse genome falls into long regions with high single-nucleotide polymorphism (SNP) rate. Genomes of laboratory mice are mosaics with the vast majority of segments derived from *Mus musculus domesticus* and *Mus musculus musculus*. (Wade et al. 2002). These observations significantly contributed to the theoretical basis of the strategy of experiments for gene mapping. However, first steps on the way of genetic studies of *Leishmania* infection control were done much earlier, before the detailed analysis of the mouse genome was performed. It was shown that inbred mouse strains had different susceptibility to *L. donovani* (Bradley et al. 1972) and *L. major* (Kellina 1973). Genetically diverse mice had differences in skin lesion development (Howard et al. 1980, De Tolla et al. 1981, Fortier et al. 1984).

1. Quantitative trait loci

Leishmaniasis and other diseases, development of which is influenced by multiple genetic factors, are quantitative traits. Genetic loci that control such conditions are defined as quantitative trait loci (QTLs). Their combined effect determines phenotype of the disease. In principle, QTL mapping is relatively simple. All that is required are two inbred strains with different alleles at loci of interest and molecular markers that are polymorphic between these strains. A mapping population of backcross, F_2 hybrids, recombinant inbred lines, chromosome substitution strains, genome-tagged mice, advanced intercross lines and recombinant congenic strains can be created of these two strains. Studying of phenotypes and multi-locus genotyping of the individuals within this population can lead to detection of the loci that control the multiple trait. One QTL can contain several genetic loci at the same chromosome (Mackay 2001).

2. Recombinant inbred strains

Recombinant inbred (RI) strains are produced by inbreeding separate pairs of F_2 hybrid mice from a cross of two strains (Bailey 1971). Each RI strain is homozygous at all loci, but contains a unique combination of 50% genes from one parental strain and 50% from the other. A group of RI strains (preferably >20) contains different combinations of genes from the two strains: the strain-distribution pattern. RI strains have proved to be a powerful tool for establishing the chromosomal position of single-gene traits. However, the capacity of RI strains to effectively establish linkage for traits that are controlled by many genes with low penetrance has not been proven. The main obstacle is the need to distinguish precisely which of the many genes that affect the phenotype is involved in each individual RI strain (Demant 2003).

3. Chromosome substitution strains

Chromosome substitution strains (also known as consomic strains) are a set of homozygous strains that are derived from two inbred parental strains. In each chromosome substitution strain, all chromosomes derive from a certain parental strain, with exception of one chromosome, which is substituted and derived from another strain. Such strains are created by selective repeated backcrossing of mice that carry one unrecombined chromosome of the donor parent to the background strain. This chromosome is recovered in at least one animal in each successive backcross generation and made homozygous after 8–10 generations. The set of chromosome-substitution strains contains one strain for each chromosome. A phenotypic difference between a background parental strain and a chromosome-substitution strain immediately maps the responsible gene(s) to the differential chromosome of this strain. This method therefore allows fast chromosomal localization of quantitative trait loci, especially when their number is limited. However, if donor and background strains differ at many QTLs, most chromosomes are likely to carry one or more

polymorphic QTLs, which make the result less informative (Naudeau et al. 2000, Demant 2003).

4. Genome-tagged mice

Genome-tagged mice (GTM) are a refined variant of chromosome-substitution strains. In this system, the substituted genetic material does not include the whole chromosome, but only a part of it (the average length is 23 cM). The GTM strains are generated in essentially the same way as the chromosome-substitution strains, but instead of selecting mice with a whole-length chromosome of the donor strain, mice are selected that only have a fragment of this chromosome. In this way, genomes of several strains with different complementary fragments can cover an entire chromosome. The group of 60 GTM strains covers a large part of the genome. Their use allows a finer resolution of the genetic control of the phenotype than the chromosome-substitution strains (Iakoubova et al. 2001, Demant 2003).

5. Advanced intercross lines

Advanced intercross lines (AILs) aim to provide a simultaneous detection and mapping of QTLs to a short chromosomal segment (Darvasi and Soller 1995). They represent an advanced generation (~F5–F8) of an intercross between two inbred strains. The animals to be crossed in each generation are selected so as to maintain maximal heterozygozity of the genome. Through the accumulation of recombinations during successive generations of intercrossing, the contiguous chromosomal segments from each parental strain become much shorter than in a standard F_2 intercross. Therefore, establishing a linkage of a QTL in AILs often also includes mapping the QTL to a short interval. However, fragmentation of the parental genomes in the AILs into short segments makes detection of linkage difficult, unless a large number of markers is used (Demant 2003). For example, AILs have been used to map susceptibility loci for trypanosomiasis (Iraqi et al. 2000) and lung tumours (Wang et al 2003).

6. Heterogeneous crosses

In contrast to the methods described above, heterogeneous crosses contain parts of genomes from several inbred strains. Any animal that is derived from a cross between two inbred strains can have only one of the two alleles at each chromosomal locus, from only one parental strain. Mice from the heterogeneous crosses might carry one of several possible alleles at each locus (Talbot et al. 1999, Mott et al. 2000). On the one hand, heterogeneous crosses offer two potential advantages. First, they maximize the number of allelic differences in the cross, and hence the number of potentially detectable QTLs. Second, they allow much faster mapping of the detected QTLs to a short interval. The chromosomes of these mice are a mosaic of short segments that are derived from different strains as a result of the recombinations that accumulate in sequential generations. This mosaic structure should allow the QTL to be localized to a single short segment. On the other hand, this approach has significant disadvantages. First, several thousands of mice is required to obtain the proper mapping precision (Mott and Flint 2002). Second, large numbers of segregating QTLs results in a larger genetic variability than in a cross of two strains. In addition, the presence of one or more QTLs of different origin that are relatively close to each other (±5–15 cM) might interfere with their recognition and with precise individual mapping. Finally, susceptibility loci may create large number of interactions that will lead to complications with the interpretation of results (Demant 2003).

7. Recombinant congenic strains

Recombinant congenic strains (RCS) model was published in 1986 (Demant and Hart 1986). The RSC are produced by inbreeding of mice of the second backcross generation between two inbread strains, one of which serves as the donor and the other as the background strain. A series of RCS consists of 20 strains, each carrying a different set of genes: approximately 12.5% genes from the common donor inbred strain, the remaining 87.5% from

the common background inbred strain. Briefly, mice of the donor strain were backcrossed twice to the background strain, and pairs of the second backcross generation (N3) were used to establish the individual RCS by an uninterrupted line of generations of brother-sister mating. In this way, the multigenic difference between the donor strain and background strain becomes transformed into a set of single gene differences between the background strain on the one hand and individual RCS on the other. Each gene contributing to the multigenic control can be mapped and studied separately using PCR-typable markers for simple sequence length polymorphism. Total three series of RCS wee produced using the background and donor strain pairs: BALB/cHeA as a background strain and STA/A as a donor strain (CcS/Dem series), C3H/DiSnA and C57BL/10ScSnA (the HcB/Dem series), and O20 and B10.O20 (the OcB/Dem series) (Demant and Hart 1986, Groot et al. 1992). The CcS/Dem series (Figure 3) was used in the experiments with *Leishmania* research that are included in the present thesis.

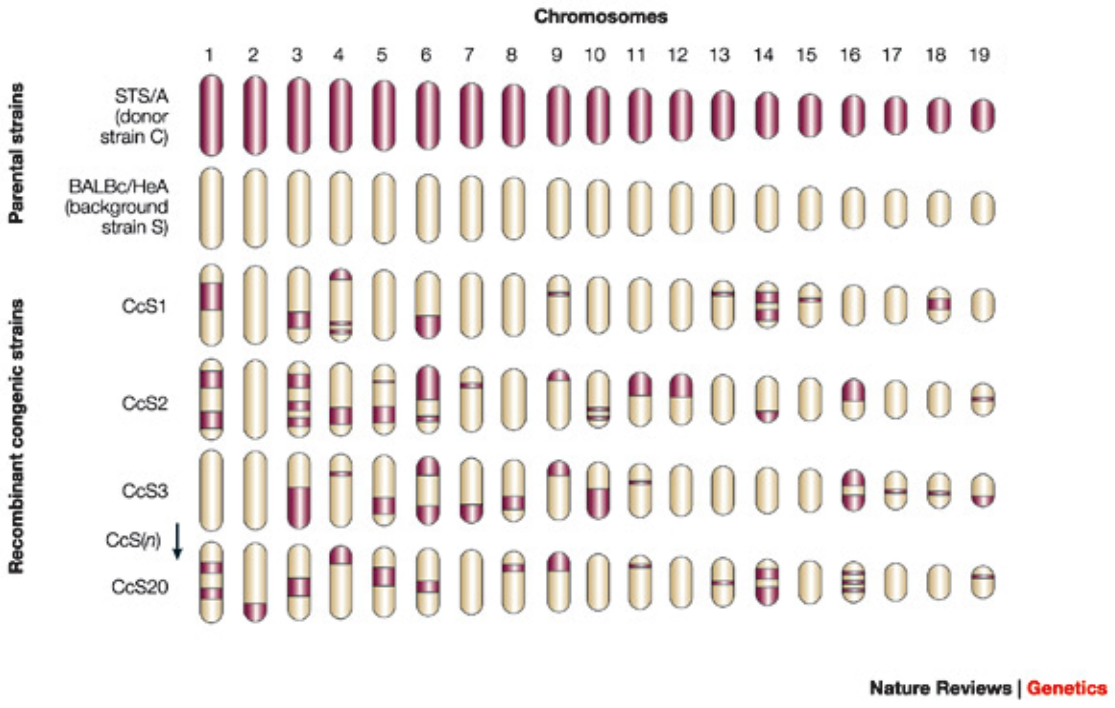
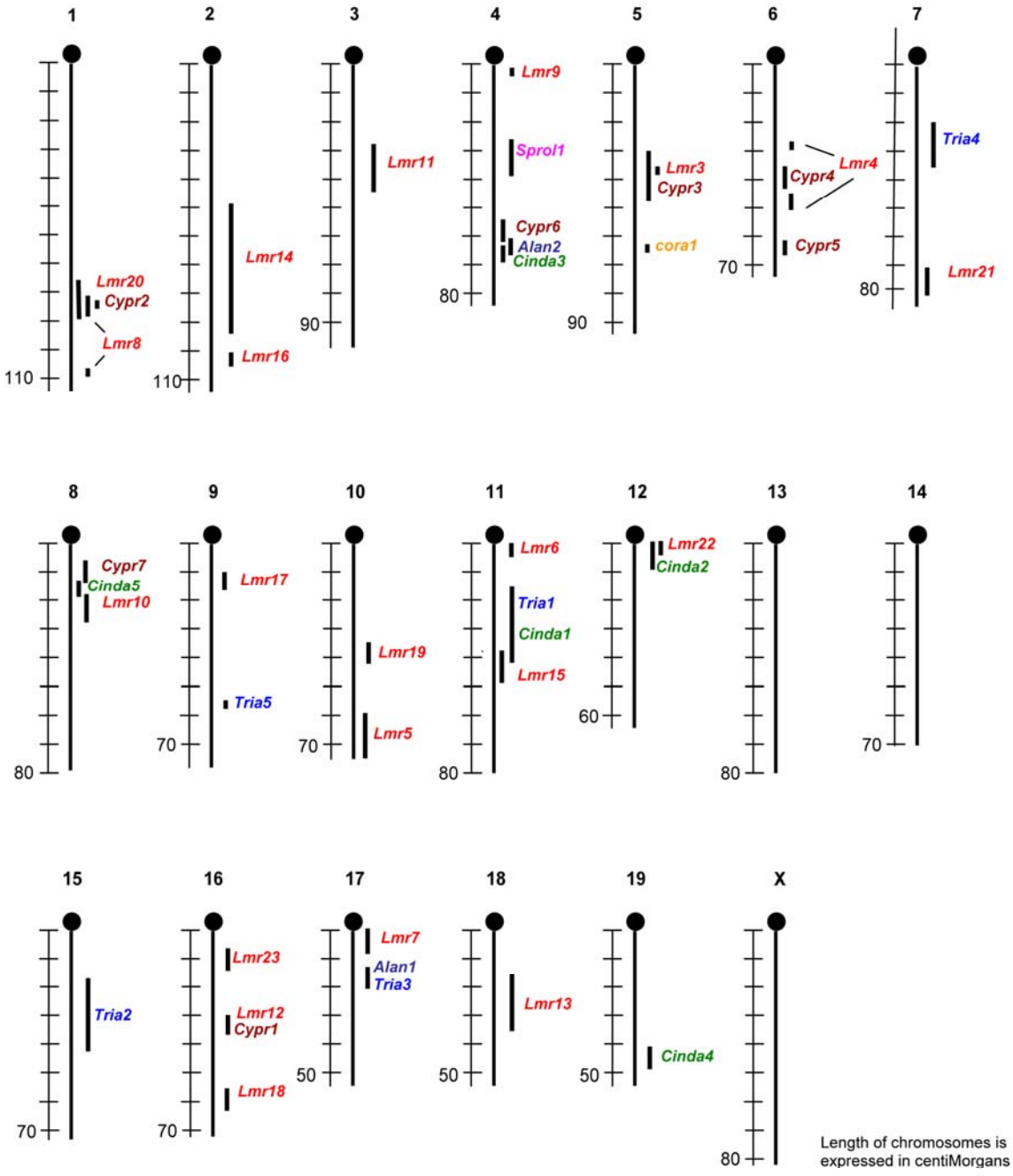


Figure 3. CcS/Dem series of recombinant congenic strains

A specific advantage of RC strains is that they contain 12.5% genes from the common donor inbred strain that are distributed on different chromosomes and each RCS is genetically well characterized, homogenous and homozygous at virtually all loci. Therefore, individual RC strains can be used to study the mechanisms of the phenotypic effects of the specific genes once they have been mapped, even if they were not yet cloned. Of specific interest to human geneticists is the application of the RCS system to speed um mapping when an approximate chromosomal location of a human gene responsible for the studied trait has been established. The information about the homologies of human and mouse chromosomes can be used to select and test RCSs that differ from the background strain at the mouse homologue of the human chromosomal region (Groot et al. 1992).

The application of RCS allowed genetic studies of various types of cancer (Demant 2003, Quan et al. 2011), cytokine-induced activation (Krulová et al. 1997, Lipoldová et al. 2005), spontaneous proliferation (Havelková et al. 1999), allogenic reactions (Havelková et al. 2000, Holáň et al. 2000), cytokine production (Kosařová et al. 1999, Lipoldová et al. 2010), T cell receptor induced activation (Havelková et al. 1996, Havelková et al. 1999), *Bordetella pertussis* infection (Banus et al. 2005), allergic asthma (Piavaux et al. 2007), and skeletal fragility (Saless et al. 2010).

Introduction of the RCS system (Figure 3) into leishmaniasis studies has stimulated significant progress in mapping of genes that are involved in susceptibility to *L. major*, which has allowed more extensive screening of segregating populations with a higher density of markers and making use of crosses with limited genetic heterogeneity. Using of RCSs have led to development of the reliable experimental model of the infection, mapping of multiple loci that determine susceptibility to various *Leishmania* species (Figure 4), and have revealed the contribution of the individual loci to different components of the immune response and disease pathology (Lipoldová and Demant 2006, Sakthianandeswaren et al. 2009; Kurey et al. 2009, Lipoldová et al. 2010, Kobets at al – in preparation).



Chromosomal location of mouse loci controlling immune response which were mapped by our laboratory

Alan **(Alloantigen response)**
Alan1 (Immunogenetics, 51: 755, 2000)
Alan2 (Genes and Immunity, 1: 483, 2000)

Cinda **(Cytokine-induced activation)**
Cinda1,2 (Genomics 42: 11, 1997)
Cinda3,4,5 (Int J Cancer 114:394-399, 2005)

cora **(correlation)**
cora1 (Immunogenetics 49: 134, 1999)

Cypr **(Cytokine production)**
Cypr1-3 (Immunogenetics 49: 11, 1999)
Cypr4-7 (Cancer Immunol Immunother 59: 203, 2010)

Lmr **(*Leishmania major* response)**
Lmr3-7 (Genes and Immunity, 1: 200, 2000)
Lmr3,5,8-14 (Genes and Immunity, 3: 187, 2002)
Lmr 13-15 (Infection and Immunity 71: 2041, 2003)
Lmr17-19 (Genes and Immunity 7: 220, 2006)
Lmr 20-23 (Immunogenetics 61:619,2009)

Sprol **(Spontaneous proliferation)**
Sprol1 (Mammalian Genome 10: 670, 1999)

Tria **(T cell receptor-induced activation)**
Tria1,2 (Immunogenetics 44: 475, 1996)
Tria 3 (Immunogenetics 49: 235, 1999)
Tria 4, 5 (Mammalian Genome 10: 670, 1999)

Figure 4. *Lmr* and other immune response loci detected in our laboratory using RCS

A range of genetic regions and candidate genes, which are involved in the control of the infection by *Leishmania*, are also shown to influence the response to other pathogens, such as *Borellia burgdorferi*, *Trypanosoma congolense*, *Salmonella typhimurium*, *Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Plasmodium chabaudi* (Lipoldová and Demant 2006).

III. Assays for the parasite detection and quantification

As with any infectious disease, spread and load of the pathogen during leishmaniasis is an important parameter, as well as precise diagnostics of *Leishmania* species. Until recently, this task was a significant complication for practical doctors and researchers. The optimal methods should be fast, simple and price-worthy. Easy-to-use techniques for rapid pilot tests during field application in the absence of the equipped laboratory are also highly necessary. The main approaches for parasite detection include DNA-based techniques, microscopy assays, cell cultures, and immunodetection (Kobets et al. 2010).

1. Microscopy for detection, quantification and histological studies

1.1. Classical and fluorescent microscopy

Microscopy assays were among the first methods for detection and quantification of a wide range of pathogens, including *Leishmania* parasites. This kind of methods allows detecting and calculating parasites directly. Histological examination of biopsies can give information about parasite load, infiltration of different cell types, formation of granulomas and other changes in *Leishmania* invaded tissues, reflecting the pathogenesis. Hematoxylin and eosin (H&E) or Giemsa are widely used for routine staining of tissue smears and biopsies (for example, Rodriguez et al. 1994, Barreca et al. 1997, Noyes et al. 1998, Abdullah et al. 1999, Ferrua et al. 2001, Weigle et al. 2002, Allahverdiyev et al. 2004, Saleem et al. 2007, Kurey et al. 2009, Kobets et al. 2010, Akhavan et al. 2010), as well as various fluorescent

labels (for example, Barreca et al. 1997, Abdullah et al. 1999, Lang et al. 2005, Lecoeur et al. 2007, Filipe-Santos et al. 2009). Microscopy belongs to the gold standard assays, being effective both for stand-alone studies (Courret et al. 2002, Lang et al. 2005, Saleem et al. 2007, Lecoeur et al. 2007) and validation of the novel methods (Noyes et al. 1998, Ferrua et al. 2001, Weigle et al. 2002, Allahverdiyev et al. 2004, Shahbazi et al. 2008, Kurey et al. 2009, Kobets et al. 2010). Among the most significant drawbacks of the microscopy assays is the fact that the direct detection of parasites on stained smears made from aspirates of bone marrow, lymph node or spleen of patients, requires invasive procedures (Spanakos et al. 2002). Microscopy analysis of large numbers of samples usually takes a lot of time. Moreover, quantification of parasites in certain samples may not reflect the real parasite load, because parasites are distributed in tissues unequally.

1.2. Electron microscopy

Interactions between *Leishmania* parasites and macrophages can be studied in details using electron microscopy. Scanning electron microscopy has revealed that the phagocytosis of *L. amazonen*sis metacyclic promastigotes begins with the engulfment of the parasite body and terminates with the progressive internalization of the flagellum. Transmission electron microscopy has revealed that promastigotes are located in very long phagosomes, in which the membrane tightly follows the outline of the parasite including that of their flagellum and immunogold cryosection electron microscopy has been useful for improving the understanding regarding the distribution and trafficking of major histocompatibility complex class II molecules in macrophages parasitized by *L. amazonensis* (Courret et al. 2002, Lang et al. 2009).

1.3. *In-vivo* imaging

The data obtained from imaging fixed samples gave a very incomplete picture of the dynamic nature of the complex processes *Leishmania* drives in both the insect and the mammalian host. Emerging technologies using fluorescence and bioluminescence imaging have been recently adapted for the study of host–*Leishmania* interactions to delineate their molecular mechanisms in the cellular context. Bioluminescence imaging of mice inoculated with transgenic *Leishmania* expressing the firefly luciferase provides an efficient and reliable method for delineating the various phases of infectious processes. Whole-body imaging with fluorescent parasites has also recently been developed to monitor light emission without substrate addition making use of different colored fluorophores for multiplex imaging. Because of its nondestructive and noninvasive nature, the procedures for bioluminescent and fluorescent imaging can be performed repeatedly, allowing each animal to be used as its own control over time, overcoming the problem of animal–animal variation (Lang et al. 2005, Lecoeur et al. 2007, Lang et al. 2009).

2. Cell culture based methods

Cell cultures include isolation and cultivation of *Leishmania* from cells and tissues. Viable parasites multiply and their numbers are evaluated after several days of incubation in the nutrient solution. This group of methods, along with microscopy assays, belongs to the first techniques developed for the estimation of parasite load in the infected host organism (Hill et al. 1983, Titus et al. 1985, Lima et al. 1997, Weigle et al. 2002, Allahverdiyev et al. 2004).

2.1. Limiting dilution assay

The majority of tissue culture techniques are based on limiting dilution assay (Titus et al. 1985, Lima et al. 1997). These methods use a range of serial dilutions to assess the ratio of

cells containing viable parasites. Limiting dilution assay, originally developed for *Leishmania* detection and quantification, is a highly laborious and time consuming technique. Detection of viable parasites is the main advantage of this method. Using only one pre-selected cell dilution combined with the direct counting of parasites may make the technique significantly less laborious and allow processing of larger numbers of samples during genetic experiments. Since culture methods target viable cells, they should be performed immediately after isolation of the tissues and require sterile conditions (Kobets et al., in preparation).

2.2. Microculture and miniculture

A microcapillary culture method (MCM) was developed for diagnosis of cutaneous leishmaniasis. In contrast to traditional culture method, MCM is more rapid, uses smaller sample, and has higher sensitivity for detection of promastigotes. In comparative studies, the average time period of incubation needed to detect of promastigotes was much shorter with the microcultures than the conventional cultivation and consisted 2-7 days versus 2-30 days. The high sensitivity of the MCM may be explained by the use of capillary tubes, wich concentrate the sample material and provide microaetophilic conditions with high CO_2 that is favorable for transformation of amastigotes to promastigotes (Allahverdiyev et al. 2004, Boggild et al. 2008). Miniculture method, in which parasites are cultivated inside the Eppendorf tubes, also showed better results than conventional culture methods (Boggild et al. 2008).

2.3. Multilocus enzyme electrophoresis (MLEE) for parasite classification

MLEE is based on the isoenzyme analysis and also requires preparation of parasite cultures. It is one of the most comprehensive methods used for identification of *Leishmania*, particularly the MON system, which was developed in Montpellier, France. MON system is based on 15 enzymes (malate dehydrogenase, malic enzyme, isocitrate dehydrogenase, 6-

phosphogluconate dehydrogenase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, NADH diaphorase, purine nucleoside phosphorylase, purine nucleoside phosphorylase, 2 glutamate-oxaloacetate transaminases 1 and 2, phosphoglucomutase, fumarate hydratase, mannose phosphate isomerase, glucose phosphate isomerase) (Rioux et al. 1990, Mauricio et al. 2006, Pratlong et al. 2009). MLEE includes *Leishmania* parasite isolation and cultivation, followed by preparation of enzyme extracts from the promastigotes pellets. Extracts are analyzed by electrophoresis. The distance that each reproducible enzyme band migrates from the origin (anode) is measured. The obtained set of bands defines the zymodeme, an electrophoretic profile for each extract. Attribution of the species is made by comparison of the profiles with the reference *Leishmania* strains (Marco et al. 2006). Isoenzymes have been used to generate phylogenetic trees and to provide a basis of the current taxonomy of *L. donovani complex*, which has three designated species, *L. donovani*, *L. infantum* and *L. archibaldi* (Mauricio 2006). For diagnostic purposes, lesion aspirates from patients were analyzed by MLEE (Marco 2006).

3. Assays that detect parasite DNA

In general, DNA-based methods were developed to detect presence of different regions of parasite DNA in experimental or clinical samples (Table 1). Techniques of this type serve two main purposes. First, they can be used for detection of *Leishmania* presence and measurement of parasite load in cells and tissues. Second, species- or subspecies-specific methods help to identify the parasite and make precise diagnosis.

Table 1. Specific targets used in DNA-based assays for detection and quantification of *Leishmania spp.*

Target	Description	References
kDNA	Minicircle kinetoplast DNA. Each parasite contains about 10 000 of minicircles.	Rodgers et al. 1990, Noyes et al. 1998, Wigle et al. 2002, Volpini et al. 2006, Kobets et al. 2010
18S rRNA / ssu-rRNA	The gene that encodes RNA included to the small ribosomal subunit.	Van Eys et al. 1992, Spanakos et al. 2002, Espinosa et al. 2009, Roelfsema et al. 2010
mini-exon	A very small gene of 39 nucleotides, present in *Kinetoplastida*. An intron, adjacent to the mini-exon, is conserved. It is followed by a sequence that varies in length considerably between species and is much less conserved.	Katakura et al. 1998, Roelfsema et al. 2010
ITS1 region	Internal transcribed spacer 1, a sequence located between the 18S ribosomal RNA and 5.8S ribosomal RNA genes.	Nasereddin et al. 2008, Roelfsema et al. 2010
ITS2 region	Internal transcribed spacer 2, a sequence located between the 5.8S ribosomal RNA and 28S ribosomal RNA genes.	Akhavan et al. 2010
7SL RNA	7SL RNA together with six proteins forms a signal recognition particle which mediates protein translocation across the endoplasmic reticulum in eukaryotes, including *Leishmania*.	Azmi et al. 2010
gp63	The gene of *Leishmania* surface protease; highly conserved.	Tupperwar et al. 2008
hsp70	The gene of 70kDa heat shock protein; highly conserved among eukaryotes, however, contains some variable regions; used for phylogenetic studies.	Fraga et al. 2010, Da Silva et al. 2010
DNA/RNA polymerases	Genes that encode DNA and RNA polymerases. Their sequences were used to reveal evolution of *Leishmania*.	Croan at al. 1997
cyt b	Cytochrome *b* gene is present in the mitochondrial genome, encodes the central catalytic subunit of an enzyme present in the respiratory chain of mitochondria; the gene was used for phylogenetic studies.	Asato et al. 2009
g6pdh	The gene of glucose-6-phosphate dehydrogenase.	Zemanová et al. 2006 Castilho et al. 2008
icd	The gene of isocitrate dehydrogenase	Zemanová et al. 2006
me	The gene encoding cytosolic NADP-malic enzyme	Zemanová et al. 2006
mpi	The gene of mannose phosphate isomerase	Zemanová et al. 2006
fh	The gene encoding fumarate hydratase	Zemanová et al. 2006
cpb	A cluster of genes for cysteine protease B.	Oshaghi et al. 2009
asat	The gene of aspartate aminotransferase	Mauricio et al. 2006
gpi	The gene encoding glucose-6-phosphate isomerase	Mauricio et al. 2006
nh1	The gene of nucleoside hydrolase 1	Mauricio et al. 2006
nh2	The gene of nucleoside hydrolase 2	Mauricio et al. 2006
pgd	The gene of 6-phosphogluconate dehydrogenase	Mauricio et al. 2006

3.1. Conventional polymerase chain reaction (PCR), nested PCR, and real-time PCR (RT-PCR) for parasite detection and quantification

PCR is an extremely sensitive tool for detection of target DNA from various sources, including *Leishmania*. Minicircle kinetoplast DNA (kDNA) contains conserved regions that are widely used for *Leishmania* detection and quantification. Each parasite contains multiple copies of kinetoplast minicircles that makes kDNA a very prominent PCR target. First PCR using primers specific to conservative regions of kDNA was tested on seven *Leishmania* species. Amplicon can be analyzes on agarose electrophoresis or dot blot (Rodgers et al. 1990, Weigle et al. 2002). Besides kDNA, a highly conserved gene *gp63* (encoding the surface protease), mini-exon (a very small gene of 39 nucleotides, present in *Kinetoplastida*), and ITS1 region (a sequence located between the 18S ribosomal RNA and 5.8S ribosomal RNA genes) were proved to be good targets both for classical PCR combined with electrophoresis and real-time PCR, but their sensitivity was either comparable or lower then the one obtained with kDNA (Nicolas et al. 2002, Bensoussan et al. 2006, Tupperwar et al. 2008, de Toledo et al. 2009, Roelfsema et al. 2010). Small ribosomal subunit gene (ssu-rRNA) was among the first targets proposed for the *Leishmania*-specific PCR assays development (Van Eys et al. 1992, Spanakos et al. 2002).

Nested PCR is based on application of two pairs of primers and includes two rounds. Additional internal primer set, specific for the particular sequences of the first amplicon, can significantly increase the sensitivity of the technique (Noyes et al. 1998, Katakura et al. 1998). For distinguishing among *Leishmania* species, the first round PCR using universal primers (for sequences that are conservative in all tested strains) should be followed by the second round PCR with specific primers that amplify the sequences that are unique for each tested strain (Akhavan et al. 2010).

Real-time PCR (RT-PCR) allows analysis of formation of the amplicon that makes it possible to quantify the initial number of template molecules. RT-PCR assays for *Leishmania* detection are based on the DNA polymerase gene, kinetoplast and ribosomal DNA. Most of these tests are not species specific. In Real-time PCR, the product is detected by the fluorescent labels (Nicolas et al. 2002, Mary et al. 2004, Rolao et al. 2004, Castilho et al. 2008). In addition to the detection and quantification, RT-PCR served for *Leishmania* species typing. Such a test was developed on basis of the *Leishmania* glucose-6-phosphate dehydrogenase locus and performed with SYBR-green or TaqMan master mixes. This enabled identification and quantification of wide range of *Leishmania* species found in the Americas. Although any amplification assay based on a single-copy target is less sensitive than the one based on multi-copy targets, the method was effective for identification and quantification of parasites in human biopsy samples (Castilho et al. 2008). SYBR-green binds nonspecifically to any double-stranded DNA what comprises the major limitation of this system. Therefore, the highly specific primers and precise optimization of the synthesis conditions are necessary. In addition, background fluorescence may diminish both sensitivity and specificity (Rolao et al. 2004). The ITS1 region is commonly used target in many different eukaryotic organisms for typing purposes. It is conserved enough to serve as a PCR target but sufficient polymorphisms to facilitate typing. The sensitivity of this RT-PCR was similar to the conventional PCR (Roelfsema et al. 2010).

As mentioned above (Table 1), a variety of targets are used for PCR. kDNA is one of the most reliable targets for *Leishmania* detection and quantification since there are ~10 000 minicircles per parasite. The sensitivity and specificity of this method were compared with two other PCR assays (mini-exon and ITS1), leishmanial culture and microscopic detection in order to validate these techniques for molecular diagnosis of cutaneous leishmaniasis. The kDNA PCR was the most sensitive diagnostic assay and should be employed when species identification is not required. However, when further parasite characterization is needed, the

ITS1 PCR is both highly sensitive and specific and enables the identification *Leishmania* species. PCR is now the diagnostic method of choice for cutaneous and mucocutaneous leishmaniasis, and kDNA PCR is the gold standard against which all new techniques should be compared (Bensoussan et al. 2006, Nasereddin et al. 2008, de Lima et al. 2010). PCR using ssu-rRNA also showed lower sensitivity than kDNA PCR (Van Eys et al. 1992, Spanakos et al. 2002).

3.2. PCR-ELISA for parasite detection and quantification

Important progress was achieved with application of PCR-ELISA, which includes conventional PCR with enzyme-linked immunosorbent assay (ELISA) for the labeled product detection. Conserved regions of kinetoplast minicircle appeared to be the preferential target used for testing (Pinero et al. 1999, Martin-Sanchez et al. 2001, Rolao et al. 2004, Alborzi et al. 2004, Kobets et al. 2010). PCR-ELISA has sensitivity comparable to RT-PCR and allows detection of small differences in parasite load. However, some steps of this technique contain drawbacks that may influence the final result. If the product is not purified, its hybridization with the biotinylated (Pinero et al. 1999, Martin-Sanchez et al. 2001) or digoxigenin-labeled probes (Rolao et al. 2004, Alborzi et al. 2008) increases the possibility of the non-complete or nonspecific binding of the probe to the amplicon or even DNA and distortion of the result. Two labeled primers can be used to omit the step of hybridization. Highly optimized reaction with sensitive kDNA primers, originally labeled with biotin and digoxygenin, excludes probability of nonspecific effects, allows precise quantitative measurement of the parasite load and fast analysis of large number of samples (Kobets et al. 2010). The method has been already successfully applied in two experimental projects (Kurey et al. 2009, Rohoušová et al. 2011).

3.3. OligoC-TesT

OligoC-TesT is a simplified and standardized PCR format for molecular detection of *Leishmania*. The test is based on PCR amplification of a small sequence of the 18S rRNA gene followed by visualization of the PCR products on a dipstick by hybridization with a gold-conjugated probe. The test gives rapid result and could be useful for diagnosis of leishmaniasis, but not for parasite quantification (Deborggraeve et al. 2008, Espinosa et al. 2009).

3.4. Restriction fragment length polymorphism (RFLP), reverse dot blot (RDB) and reverse line blot (RLB) for *Leishmania* species identification

A spectrum of different species can be found in patients sent to the diagnostic laboratories. Since the therapeutic strategy depends on the *Leishmnaia* species involved, it is the task of great importance to type the infecting parasite in a sensitive way. The most commonly used targets are 18S ribosomal RNA gene, the 39 nucleotides small mini-exon gene, and ITS1 region between the 18S ribosomal RNA and 5.8S ribosomal RNA genes. All these regions are highly repetitive and conservative, though a part of their sequences vary that allows using them for species typing. Mini-exon gene is unique and tandemly repeated in *Kinetoplastida*, but is absent in mammalian hosts and sand fly vectors. An intron, adjacent to the mini-exon, is conserved. It is followed by a sequence that varies in length considerably between species and is much less conserved. Primers were targeted both to the conserved mini-exon and its intron in order to amplify the polymorphic intergenic region (Fernandes et al. 1994, Cupolillo et al. 1995, Katakura et al. 1998, Marfurt et al. 2003, de Toledo et al. 2009, Roelfsema et al. 2010).

The studies that compared different targets (18S ribosomal RNA gene, mini-exon and ITS1 region) revealed that the highest sensitivity was achieved with the primer for ITS1. Often the ITS1 gave clearer PCR products when compared to the mini-exon results

(Roelfsema et al. 2010). Compared to kinetoplast DNA (kDNA), which is considered to be the most sensitive PCR approach for *Leishmania* parasite detection, ITS1 and mini-exon PCR methods suffered from reduced sensitivity, especially when tested on clinical material collected on filter papers (Bensoussan et al. 2006).

7SL RNA was used as a target for detection of Old World *Leishmania* Species. 7SL RNA together with six proteins forms a signal recognition particle which mediates protein translocation across the endoplasmic reticulum in eukaryotes, including *Leishmania*. Species-specific RFLP patterns were obtained when the PCR product was digested with the restriction enzymes. Results were compared with reverse dot blot (RDB) assay, for which biotin-labelled amplicons were hybridized to specific oligonucleotide probes immobilized onto a membrane. It increased the sensitivity by tenfold compared to the detection of PCR products by gel electrophoresis (Azmi et al. 2010).

Biotinilated ITS1-specific PCR primers and reverse line blot (RLB) assay were used for evaluation of 21 reference strains of several *Leishmania* species. Similarly to RDB, biotinilated PCR product hybridize specifically to oligonucleotide probes coupled to a membrane support. Using appropriate hybridization conditions, it is possible to differentiate between amplicons whose DNA sequences differ by only one nucleotide. The sensitivity of the RLB was comparable to that using kDNA PCR (Nasereddin et al. 2008).

Though the gene *hsp70* is conserved across prokaryotes and eukaryotes, nucleotide sequence variation is sufficient to discriminate parasite species (Fraga et al. 2010). Analysis of *hsp70* gene sequence led to the identification of restriction enzymes that could also be used for PCR-RFLP based identification (da Silva et al. 2010).

3.5. Sequencing for the pathogen classification and phylogenetic studies

A single amino acid polymorphism can be responsible for change in enzyme mobility, but some distinct genotypes produce phenotypes, indistinguishable using MLEE, described

above (Mauricio et al. 2006). Though RFLP analysis also proved to be a reliable method for typing of *Leishmania* species, it was not optimal for *L. Viannia* subgenus (also known as *L. braziliensis* complex) detection. In such cases, direct sequencing of the product solved the problem. Reference strains should be used to assign the samples to the various known species (Roelfsema et al. 2010).

Analysis of DNA and RNA polymerases coding genes has the potential for use in genetic typing and population genetics, their gene sequences were analyzed to reveal the evolution of the genus *Leishmania* (Croan et al. 1997). Multilocus sequence typing, which includes amplification and sequencing of polymorphic regions (Table 1), appeared to be a very informative method for investigation of relationships between zymodemes, and more precise parasite identification and description, enhancing MLEE (Mauricio et al. 2006, Zemanova et al. 2006).

Other DNA targets were also used for investigation of the *Leishmania* phylogeny. Species identification can be based on sequencing of the cytochrome *b* (c*yt b*) gene. It is contained in the mitochondrial genome of a wide variety of living forms and encodes the central catalytic subunit on an enzyme present in the respiratory chain of mitochondria. This gene has been widely used for phylogenetic studies and identification of animals and plants (Luyo-Acero et al. 2004). Using cytochrome *b* gene as a target allowed to explore *Leishmania* species/subspecies phylogenetic relations, and facilitated detection of 28 *Leishmania* strains in samples from patients (Luyo-Acero et al. 2004, Asato et al. 2009).

Besides using for RFLP, mentioned above, the gene encoding 70 kDa heat-shock protein (*hsp*70 and HSP70 respectively) was sequenced for studying evolutionary relationships of 16 *Leishmania* species from the Old and New World, This target has also been applied in phylogenetic studies of different parasites (Fraga et al. 2010).

3.6. Polymorphism specific PCR (PS-PCR)

On the basis of knowledge of the c*yt b* gene sequence, a polymorphism specific PCR procedure was developed. Results were visualized using agarose gel (Marco et al. 2006). Discriminative PCR focusing on the cathepsin-1 proteases and targeting the *cbp* gene was first used to confirm the taxonomic status of the specimens if they belong to *L. donovani* complex (*L. infantum* and *L. donovani*). However, for more precise detection, the product should be processed with the restriction enzymes for species specific sites (Hide et al. 2006, Oshaghi et al. 2009).

The combination of PS-PCR with two subgenus-specific PCR allows detection of major *Leishmania* species causing New World cutaneous leishmaniasis in formalin fixed biopsy samples. The samples are easily transported without freezing, what makes the technique beneficial for diagnostics (Mimori 1998).

4. Immunodetection

Enzyme-linked immunosorbent assay (ELISA) is based on indirect detection of parasites in the infected host by the presence of *Leishmania* antigens (Ferrua et al. 2001) or antibodies to them (Lima et al. 2003), using anti-*Leishmania* antibodies and total antigen from lyzed promastigotes, respectively. Immunological cross-reaction of *Leishmania* with *Trypanosoma cruzi*, which is co-endemic in some areas, has posed a serious problem in the use of serodiagnostic tests. However, specific monoclonal antibodies against *Leishmania* could solve this problem (McMahon Pratt et al. 1981). One of the important advances in the indirect diagnosis of leishmaniasis was the production and identification of recombinant antigens. Specifically, the antigen rK39, a recombinant protein, resembles a part of a 230-kDa protein of the kinesin family, coded by the kinetoplast DNA of *Leishmania*. Reactivity with the other trypanosomatids is negative and the presence of the antibody, anti-rK39, indicates an active infection. Comparison between immunochromatographic rK39 test and ELISA

exhibited no significant differences (de Lima et al. 2010), nevertheless Western blot test was found to be more sensitive and suitable for the serodiagnosis of anthroponotic cutaneous leishmaniasis than conventional ELISA (Zeyrek et al. 2007).

Estimation of the presence of *Leishmania* antigens and anti-*Leishmania* antibody levels need not reflect the real parasite load because the presence of antibodies to parasites highly depends on the individual responsiveness and general health condition of the host. Due to this fact, the diagnosis, obtained with immunodetection-based assays, should be confirmed by other methods.

5. Rapid tests

A separate group of assays create rapid noninvasive diagnostic methods, which detect the presence of antibodies to *Leishmania* in the blood, serum or urine. Recombinant K39 dipstick was successfully used in the field. One-two drops of blood followed by one-two drops of buffer were placed on the pad of the dipstick and incubated for approximately 5 minutes. The test was positive when two bands, a control band and a test band, appeared within 5 minutes (Bern et al. 2000). The direct agglutination test (DAT) can be performed either on blood or serum, measuring the titer of the immune complexes. The threshold titer chosen to define a positive test is crucial for proper analysis (Bern et al. 2000). Latex agglutination test (LAT) is based on the detection of a leishmanial antigen in urine. In patients with visceral leishmaniasis, it was proved to be useful in the treatment monitoring (Vilaplana et al. 2004).

6. Leishmanin skin test (LST)

It includes intradermal administration of *Leishmania* antigen followed by the induration measurement. It is used as an indicator of cell-mediated immunity against *Leishmania* parasites *in vivo*. Leishmanin inoculation into the skin of an individual in whom

previous infection has led to a state of cell-mediated immunity will result in a delayed-type hypersensitivity reaction characterized by erythema and induration at the site of antigen injection after a few hours. LST was used as a marker of previous exposure to *Leishmania* parasites in clinical and epidemiological studies, but showed lack of sensitivity and required improvement. Moreover, LST has significant limitations in case of visceral leishmaniasis, because patients with active disease are in a state of anergy and unresponsive to the test (Sassi et al. 1999, Bern et al. 2006, Gidwan et al. 2009).

7. Which method is better for the particular study and how to choose it?

Taking into consideration the wide range of the methods that were developed during relatively long period of time (Table 2), the selection of the most suitable technique sometimes may become a quite complicated question.

Microscopy detection of parasites in Giemsa or hematoxylin and eosin (H&E) stained samples considered to be the classical standard from the beginning of *Leishmania* studies. Though these methods are laborious, time consuming and lack sensitivity, they are highly specific, visualize the parasite directly, and remain a perfect tool for illustration of the result.

Until recent, the limiting dilution assay was commonly used for parasite quantification. This method has similar limitations as microscopy, requiring a lot of time and work; moreover, all culture-based techniques are highly dependent on sterile conditions. It can take days or weeks until parasites are observed, depending on the species and number of parasites seeded. Moreover, the cultures may be contaminated. These characteristics nearly exclude the possibility of it being used for routine examinations of biopsy specimens collected under field conditions from patients (Bensoussan et al. 2006, Castilho et al. 2008). Nevertheless, culturing of parasites can reflect their viability and ability to multiply.

At the present moment, the DNA-based methods create the most popular and intensively developing group of assays. They were proved to be reliable, time-saving, and

most suitable for the purposes of diagnostics and research, especially for analysis of large numbers of samples. These significant advantages over the other methods make them a powerful tool for *Leishmania* parasites detection, quantification and identification of species and subspecies.

Independently on the target, real-time PCR is the preferred method for many diagnostic labs because it is fast and provides the opportunity to perform a multiplex PCR. However, inhibition of the PCR can sometimes be seen, particularly with skin biopsies, and leads to a lower rise of the curve during the reaction (Roelfsema et al. 2010). Because of using fluorescent probes, the price of RT-PCR is usually a bit higher than for other PCR-based assays. Moreover, modern specialized RT-PCR machines and software required for this method are still not affordable for many clinical and scientific labs in developing countries.

In this aspect, PCR-ELISA is cheaper than RT-PCR and it is based on robust approaches, routinely used for a relatively long time. Common PCR machines and ELISA readers are available in laboratories all over the world, because these techniques are widely used for research and clinical purposes. Sensitivity of PCR-ELISA is comparable to that of RT-PCR. These advantages make PCR-ELISA a preferential method in many cases.

In a number of cases, precise identification of the *Leishmania* species is necessary for the clinicians to make correct decisions on appropriate therapy. Such information is also valuable for the epidemiological studies. For a long period of time, the main assay for *Leishmania* species identification was MLEE. However, this method has some significant disadvantages. Besides the risk of the bacterial contamination during cultivation, the parasite strains with the same enzyme phenotype may in fact have distinct amino acid sequences (Mauricio et al. 2006, Foulet et al. 2007). Cytochrome b gene sequencing proved to be an effective and useful method for phylogenetic analysis of *Leishmania* genus (Luyo-Acero 2004, Asato 2009). Nevertheless, similarly to MLEE, it needs cultivation of parasites that implicates the same drawbacks as all cell culture based methods, described above.

When scientist meet problems with detection of *Leishmania* species with MLEE, PCR assays using primers against polymorphic regions can help. Combination of these techniques with RFLP or RDB/RLB significantly increases their sensitivity, helping to escape false negative diagnosis. Digestion of the PCR product with restriction enzymes allows identification of almost all pathogenic *Leishmania* species, thus enabling direct rapid characterization of the infecting parasite (Nasereddin et al. 2008, Azmi et al. 2010). Results from polymorphism specific PCR have agreed both with *cyt b* gene sequencing and MLEE. Therefore, all these techniques could have an application on the identification of the *Leishmania* (Marco et al. 2006).

Noninvasive rapid tests (LAT, DAT, rK39 dipstick test) deserve special attention, because they are commercially available, easily applicable and give fast results. In the comparative study, rK39 dipstick test was less sensitive than DAT, but was easier to use and results could be obtained within minutes (Bern et al. 2000). In other studies, these tests showed comparable sensitivity and were more reliable than LAT (Singh et al. 2010). Noninvasive rapid tests share the main disadvantage of all immunodetection assays: their results are highly dependent on the individual immune response of the organism, what may cause discrepancies in diagnostics and the presence of false negative samples. Despite of it, such tests could be especially valuable in the field conditions, for mass-surveys, as well as for confirmation of clinically suspected cases.

All advantages and disadvantages of the methods, described above, will determine their wide possibilities of use both for experiments and clinical practice. Finally, it is worth to mention again that all novel methods always need standardization and quality control. For this purpose, results obtained using the newly developed techniques must be compared with results from the classical ones (microscopy, kDNA PCR, MLEE) to test the efficacy of the fresh approaches.

As we can conclude, there is no one universal method that could be used in any case when the parasite load is necessary to be measured. Due to their advantages and disadvantages, all approaches have different perspectives of use. PCR-based techniques are the most sensitive at the present moment, so we highly concentrated our attention on them. PCR-ELISA is robust, relatively simple and cheaper method in comparison with other quantitative assays. Taking into account these facts, we decided to design an improved PCR-ELISA. Rising the advantages and reducing disadvantages of the method, we made it more optimal for the purposes of our study, and also increased its potential of application by other laboratories. In addition, we continue using different, non-PCR-based techniques, as we have multiple approaches for the research of leishmnaiasis.

Table 2. Summary of the methods for *Leishmania* parasite detection

Assay		*Leishmania* species	Target	Sensitivity (lowest detectable parasite load)	Samples	Reference
Micro-scopy	Giemsa staining	*L. mexicana* *L. braziliensis*	parasite cells	N/D	skin biopsies	Rodriguez et al. 1994
	Acridin orange staining	*L. donovani*	parasite cells	N/D	parasite culture	Barreca et al. 1997
	H&E staining	*L. tropica*	parasite cells	N/D	skin biopsies	Saleem et al. 2007
	Electron microscopy	*L. amazonensis* *L. major*	parasite cells	N/D	macrophages	Courret et al. 2002
	In-vivo imaging	*L. amazonensis* *L. major*	parasite cells	N/D	whole mouse	Lang et al. 2005 Lecoeur et al. 2007
Culture-based methods	Limiting dilution assay	*L. major*	viable parasites	N/D	skin biopsies	Lima et al. 1997
	Limiting dilution assay	*L. major*	viable parasites	N/D	lymph nodes, spleen	Kobets et al. in preparation
	Microcapillary culture method (MCM)	*L. tropica*	viable parasites	N/D	skin biopsies	Allahverdiyev et al. 2004
	Microcultures and minicultures	*Leishmania spp.*	viable parasites	N/D	lesion aspirates	Boggild et al. 2008
	Multilocus enzyme electrophoresis (MLEE)	*L. major* *L. tropica*	12 enzymes	N/D	lesion aspirates	Marco et al. 2006
DNA-based methods	Polymerase chain reaction (PCR)	*L. braziliensis* *L. mexicana* *L. major* *L. chagasi* *L. donovani* *L. aethiopica* *L. enriettii*	kDNA	0.1-10 fg	parasite DNA, skin biopsies	Rodgers et al. 1990
	PCR + dot blot hybridization	*Leishmania (Viannia)*	kDNA	0.1 fg	parasite DNA, skin biopsies, lestion aspirates, lesion scraping	Wigle et al. 2002
	PCR	*L. infantum* *L. donovani*	ssu-rRNA gene	10 parasites in 1 ml	parasite DNA, blood	van Eys et al. 1992, Spanakos et al. 2002

	PCR	*L. braziliensis* *L. amazonensis*	kDNA	N/D	Giemsa stained slides	Volpini et al. 2006
	Nested PCR	*L. tropica*	kDNA	0.1 fg	parasite DNA, skin biopsies	Noyes et al. 1998
	Nested PCR	*L. donovani*	mini-exon	100 fg	parasite DNA, tissue aspirates	Katakura et al. 1998
	Nested PCR	*L. major* *L. gerbilii* *L. turanica*	ITS2	N/D	parasite DNA, ear biopsies	Akhavan et al. 2010
	Real-time PCR (RT-PCR)	*L. major* *L. donovani* *L. amazonensis* *L. mexicana*	kDNA	100 fg	parasite DNA	Nicolas et al. 2002
	Real-time PCR (RT-PCR)	*L. braziliensis* *L. peruviania* *L. guianensis* *L. panamensis* *L. lainsoni* *L. utingensis* *L. amazonensis* *L. mexicana* *L. pifanoi* *L. infantum* *L. adleri*	*g6pdh*	10 parasites in 0.2 ng of total DNA	parasite DNA, skin biopsies, mice footpad	Castilho et al. 2008
	Real-time PCR (RT-PCR)	*L. donovani* *L. major* *L. chagasi* *L. mexicana* *L. braziliensis*	*gp63*	10 fg	parasite DNA	Tupperwar et al. 2008
	Real-time PCR (RT-PCR)	*L. donovani*	ITS1	10 fg	parasite DNA	Roelfsema et al. 2010
	PCR + enzyme-linked immunosorbent assay (PCR-ELISA)	*L. braziliensis*	kDNA	0.5-0.6 pg	parasite DNA, blood	Pinero et al. 1999
	PCR-ELISA	*L. infantum*	kDNA	1 fg	parasite DNA, skin biopsies	Martin-Sanchez et al. 2001
	PCR-ELISA	*L. major*	kDNA	0.3 fg	parasite DNA	Kobets et al. 2010
	Oligo-C-TesT	*L. guyanensis* *L. braziliensis*	18S rRNA gene	N/D	lesion scrapings	Espinosa et al. 2009
	Restriction fragment length polymorphism (RFLP)	*L. donovani* *L. infantum* *L. tropica* *L. aethiopica* *L. braziliensis*	18S rRNA gene	100 fg	parasite DNA, skin biopsies, blood, bone marrow	Roelfsema et al. 2010
			Mini-exon	50 fg		
			ITS1	10 fg		

	RFLP + reverse dot blot (RDB)	*L. donovani* complex *L. major* *L. tropica* *L. aethiopica*	7SL RNA gene	20 fg	parasite DNA, skin biopsies	Azmi et al. 2010
	Reverse line blot (RLB)	*L. donovani* *L. major* *L. tropica* *L. aethiopica* *L. infantum*	ITS1	60 fg – 5 pg, depending on prpbe	parasite DNA, skin biopsies	Nasereddin et al. 2008
	Sequencing	19 species	DNA and RNA polymerase genes	N/D	parasite DNA	Croan et al. 1997
	Multilocus sequence typing	*L. donovani* *L. infantum* *L. archibaldi*	*asat* *gpi* *nh1* *nh2* *pgd* *d6pdh* *icd* *me* *mpi* *fh*	N/D	parasite DNA	Mauricio et al. 2006, Zemanova et al. 2006
	Sequencing	24 species	*cyt b*	N/D	patasite DNA, clynical isolates	Asato et al. 2009
	Sequencing	*L. Viannia* sp. (*L. braziliensis* complex)	mini-exon	50 fg	parasite DNA, tissue samples	Roelfsema et al. 2010
	Sequencing	16 species	*hsp70*	N/D	parasite DNA	Fraga et al. 2010
	Sequencing + RFLP	*L. guyanensis L. braziliensis* *L. lainsoni* *L. shawi* *L. naiffi* *L. amazonensis* *L. infantum* *L. utingensis* *L. lindenbergi*	*hsp70*	N/D	parasite DNA	Da Silva et al. 2010
	Polymorphism-specific PCR (PS-PSR)	*L. braziliensis* *L. guyanensis* *L. panamensis* *L. mexicana* *L. amazonensis*	polymorphic regions	N/D	parasite DNA, clinical isolates	Marco et al. 2006

	Species-specific PCR + RFLP	*L. infantum* *L. donovani*	*cpb*	N/D	patasite DNA	Oshaghi et al. 2009
	Polymorphism-specific PCR (PS-PCR)	*L. panamensis* *L. braziliensis* *L. guyanensis* *L. mexicana* *L. amazonensis*	polymorphic regions	N/D	parasite DNA, formalin-fixed biopsy samples	Mimori et al. 1998
Immuno-detection	ELISA	*L. infantum*	*Leishmania* antigen	N/D	spleen, liver	Ferrua et al. 2001
	ELISA	*L. chagasi*	anti-*Leishmania* antibodies	N/D	cerebrospinal fluid	Lima et al. 2003
	Western blot	*L. infantum*	anti-*Leishmania* antibodies	N/D	serum	Zeyrek et al. 2007
	Latex agglutination test (LAT)	*Leishmania spp*.	*Leishmania* antigen	N/D	urine	Vilaplana et. al 2004
	Recombinant K39 test (rK39)	*Leishmania spp*	anti-*Leishmania* antibodies	N/D	blood	Bern et al. 2000
	Direct agglutination test (DAT)	*Leishmania spp*	anti-*Leishmania* antibodies	N/D	blood, serum	Bern et al. 2000
Delayed-type hyper-sensitivity reaction	Leishmanin skin test (LST)	*L. major*	cell-mediated immune responce	N/D	skin	Sassi et al. 1999

Aims of the study

I. Design, optimization and application of different assays for detection and quantification of *Leishmania* parasites

1. Development of the improved PCR-ELISA method for quantitative measurement of parasite load in tissues of the infected organism.

2. Modification of the limiting dilution assay that would allow processing of a large number of samples in one experiment.

3. Using of microscopy assays as a classical standard for validation of novel methods and a visible illustration of presence of *Leishmania* in organs and tissues.

II. Estimation of parasite burden in the infected mice for characterization of the immune response during leishmaniasis

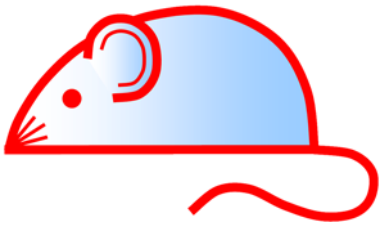
1. Detection of genetic loci that control *Leishmania major* dissemination in the infected organism.

2. Establishment of the mouse model of *Leishmania tropica* infection using genetically different RC strains.

3. Study of the influence of sand fly saliva on the development of leishmaniasis in a host.

Materials and Methods

I. Animals



Males and females of strains BALB/cHeA (BALB/c), STA/A (STS) and selected RC strains (Demant et al. 1986, Stassen et al. 1996) were tested. When used for these experiments, RC strains were in more than 38 generation of inbreeding and therefore highly homozygous. The parts of their genome inherited from the BALB/c or STS parents were defined (Stassen et al. 1996). The mice were 8 to 24 weeks old at the time of infection. During the experiment male and female mice were placed into separate rooms and males were caged individually. Mice were maintained in the animal facilities of the Institute of Molecular Genetics, v.v.i. and Charles University in Prague. The research had complied with all relevant European Union guidelines for work with animals.

Mice of the strains BALB/c (14 males) and STS (17 males) were analyzed during development of the method for parasite detection using PCR-ELISA (Kobets et al. 2010).

To detect genetic loci that control different disease manifestations in genetically diverse individuals, males and females of BALB/c parental strain, RC strain CcS-11 and 299 F_2 hybrids between CcS-11 and BALB/c (155 females and 144 males) were produced at the Institute of Molecular Genetics. The age of mice was 9 to 13 weeks at the time of infection, mean age 11 weeks, median 12 weeks. F_2 were tested in three successive experimental groups (Kurey et al. 2009).

For the study of the influence of immunization with sand fly saliva on development of liehsmaniasis, 30 female BALB/c mice (4 weeks old) were divided into five groups, immunized with sand fly saliva and sacrificed 7 weeks after the infection. In total, mice were followed for 37 weeks (Rohoušová et al. 2011).

In the experiment with *Leishmania tropica*, mice of the strains BALB/c (18 females, 12 males), STS (12 females, 13 males), CcS-3 (12 females, 13 males), CcS-5 (17 females, 12 males), CcS-11 (17 females, 19 males), CcS-12 (12 females, 6 males), CcS-16 (17 females, 12 males), CcS-18 (8 females, 12 males), and CcS-20 (12 females, 12 males) were infected with *L. tropica*. Mice were tested in 3 successive experimental groups and were killed 21, 32 and 43 weeks after infection. Age of the mice at the time of infection was 9 to 26 weeks (mean 16 weeks, median 16 weeks). In the experiments with *Leishmania major*, mice of the strains BALB/c (25 females, 26 males), STS (8 females, 9 males), CcS-3 (10 females, 10 males), CcS-5 (23 females, 29 males), CcS-11 (18 females, 20 males), CcS-12 (19 females, 16 males), Ccs-16 (11 females, 13 males), CcS-18 (9 females, 4 males), and CcS-20 (13 females, 15 males) were infected with *L. major*. Mice were tested in 8 successive experimental groups and were killed 8 weeks after infection. Age of the mice at the time of infection was 8 to 47 weeks (mean 16 weeks, median 15 weeks) (Kobets et al., in preparation).

II. Infection

Parasites *Leishmania major* LV 561 (MHOM/IL/67/LRC-L137 JERICHO II) was maintained in rump lesions of BALB/c females. Amastigotes were transformed to promastigotes using SNB-9 (Diamond et al. 1954). Promastigotes (10^7) from 6-day-old subculture 2 (Figure 5) were inoculated in 50 μl sterile saline s.c. into the mouse rump. Mice were killed 8 weeks after infection (Kurey et al. 2009, Kobets et al. 2010, Kobets et al., in preparation).

Leishmania tropica from Urfa, Turkey (MHOM/1999/TR/SU23) was used for infecting mice. Stationary phase promastigotes (10^7) from subculture 2 have been inoculated in 50 μl of sterile saline s.c. into the tail base, with promastigote secretory gel (PSG) collected from *L. tropica*-infected *Phlebotomus sergenti* females (laboratory colony originating from

Urfa focus). PSG was collected as described (Rogers et al. 2004). The amount corresponding to one sand fly female was used per mouse (Kobets et al., in preparation).

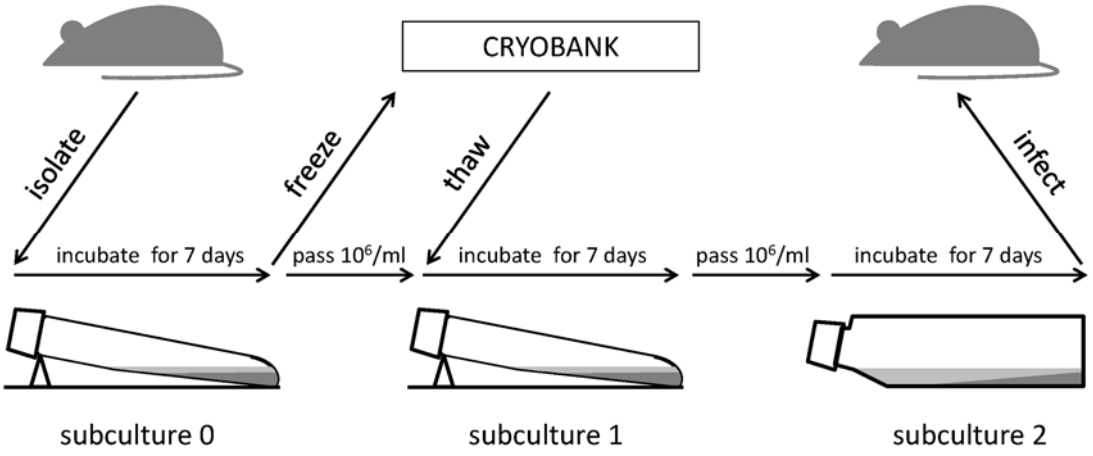


Figure 5. Preparation of parasites for inoculation ((C) Igor Grekov)

For immunization with sand fly saliva, mice were exposed to *Phlebotomus duboscqi* females (colony originating from Senegal). At each exposure, 30 female sand flies were allowed to feed on whole mouse body (for detailed exposure schemes see Rohoušová et al. 2011, Fig. 1A) with an average of 27 fed sand flies per mouse. *Leishmania* infection was monitored in BALB/c mice exposed to *P. duboscqi* bites following four different exposure schemes (Rohoušová et al. 2011, Fig. 1A). Groups 2 and 15 represented short - and long-term exposures during the sand fly season with subsequent *Leishmania* transmission. Schemes used for groups 2 + 0 and 15 + 0 mimicked short - and long-term exposures followed by a sand fly-free period and *Leishmania* transmission occurring during the subsequent sand fly season. The mice were subsequently infected intradermally in the right ear pinna with 10^4 *L. major* promastigotes (MHOM/IL/67/LRC-L137 JERICHO II) together with 1/4 gland pair equivalent of *P. duboscqi* salivary gland homogenate in 5 µl sterile saline (Rohoušová et al. 2011).

III. Disease phenotype

To determine disease phenotype, size of the skin lesions was measured weekly using a Vernier caliper gauge. Mice were sacrificed from 7 to 43 weeks after infection; the details of duration for each experiment are described above (Materials and methods, section I – Animals). Inguinal lymph nodes (Kobets et al. 2010), and also blood, spleen and liver were collected for the further analysis (Kurey et al. 2009, Kobets et al., in preparation). In the experiment with sand fly saliva, blood, infected ears and lymph nodes were isolated (Rohoušová et al. 2011).

Splenomegaly (enlargement of the spleen) and hepatomegaly (enlargement of the liver) were calculated as organ-to-body weight ratio × 1000.

IV. Quantification of parasite load in spleens and inguinal lymph nodes

In total, three different approaches were used to detect and quantify *Leishmania* parasites in the infected mice tissues: light and fluorescent microscopy, modified limiting dilution assay and PCR-ELISA.

1. Detection of *Leishmania* in tissue smears using microscopy assays

1.1. Counting of parasites in Giemsa stained tissue smears

Smears were prepared from inguinal lymph nodes and stained with Giemsa (the stain was 20 times diluted and applied for 30 minutes). *Leishmania* was quantified as the mean amastigote number from 100 microscopy fields observed (Kobets et al. 2010).

1.2. *Leishmania* detection in H&E stained tissue smears

Inguinal lymph nodes and spleens were fixed in 4% formaldehyde for 24-48 hours, dehydrated and embedded in paraffin using an automatic embedding machine. Staining of

parasites was performed in 5 μm lymph node sections. Slides were deparaffinized with xylene (2 times for 5 min) and rehydrated with 96 % ethanol (3 times for 3 min), 80 % ethanol (3 min), 70 % ethanol (3 min) and PBS (phosphate buffer saline, 3 min). Sections were washed in PBS (10 min) and stained using standard H&E procedure. Samples were examined using a microscope Leica DM 6000 (Leica, Germany) under x1000 magnification (Kurey et al. 2009, Kobets et al., in preparation).

1.3. Immunohistochemical parasite staining

After fixation and preparation of the sections as described above, parasites were stained using anti-*Leishmania* lipophosphoglycan monoclonal mouse IgM antibody (Code Nr. CLP003A, Cedarlane, Canada) diluted 1:100 in PBS with 1 % BSA (bovine serum albumine, Sigma-Aldrich, USA) and applied for 1 h at 37 °C in the biological thermostat BT 120 M (Labsystems, Finland), followed by TRITC-conjugated goat anti-mouse IgM (Code Nr. 115-025-020, Jackson Immunoresearch, USA), also diluted 1:100 in PBS with 1 % BSA and applied for 1 h at 37 °C. Cell nuclei were stained with DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) 10 ng/μl (Sigma-Aldrich, USA). Samples were examined using a microscope Leica DM 6000 under x1000 magnification (Kobets et al. in preparation).

2. Quantification of *Leishmania* using PCR-ELISA

Preparation of DNA from parasites and from analyzed tissues was performed by DNA isolation using TRI Reagent® solution (Molecular Research Center, USA) according to the manufacturer's protocol http://www.mrcgene.com/tri.htm (Kurey et al. 2009, Kobets et al. 2010, Rohoušová et al. 2011) or by a standard proteinase procedure (Laird et al. 1991, Kobets at al. 2010).

The detailed ready-to-use protocol of PCR-ELISA (Figure 6), including all solutions preparation, is given in the Nature Protocols paper (Kobets et al. 2010) that is included to this

thesis. The following primers were used in PCR-ELISA for amplification of the 120-bp conservative region of the minicircle kinetoplast of *Leishmania* parasites: digoxigenin-labeled F 5'-ATT TTA CAC CAA CCC CCA GTT-3' and biotinlabeled R 5'-GTG GGG GAG GGG CGT TCT-3' (VBC-Genomics, Biosciences Research, Austria; the sequences were obtained from Rodgers et al. 1990.

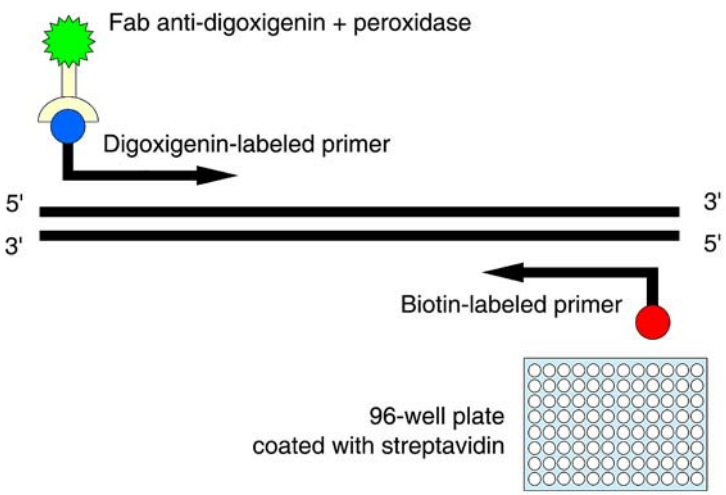


Figure 6. PCR-ELISA scheme

Briefly, 50 ng of extracted DNA with 5 pmol of each kinetoplast primer, 0.5 mM concentration of each dNTP, 2 mM MgCl2, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), and 2 U of Taq polymerase (Invitrogen, Brazil) was amplified in a total volume of PCR reaction of 20 µl. As a positive control, we used 20 ng of *L. major* DNA per reaction (0.3 fg of *L. major* DNA approximately corresponds to one parasite). After an initial hot start at 94°C for 90 s, the conditions used for amplification were: 94°C for 30 s, 53°C for 45 s, 72°C for 60 s (26 cycles for DNA from lymph nodes and 33 cycles for DNA from spleens), followed by the final extension 72°C for 10 min, using the DNA Engine DYAD Peltier Thermal Cycler or I-Cycler (Bio-Rad, USA). Concentration of the amplified 120-bp region of *Leishmania* DNA in PCR product was determined using the modified ELISA protocol (Pharmingen, USA). Plates were coated with 100 µl per well streptavidin solution (0.5 µg/ml in 0.1 M $NaHCO_3$ pH 7.0) at 4°C overnight. PCR samples were diluted to 2% concentration in 2% FCS/PBS. The positive PCR control was used as a standard for ELISA. Plates were incubated at room temperature for 2 h and then incubated at room temperature for 45 min with the anti-digoxigenin detecting antibody, Fab fragments (Roche Diagnostic, Germany) 0.15 U/ml, diluted in 2% FCS/PBS. As a substrate for color reaction development, we used 2,2′- azino-bis(3-ethylbenzo-thiazoline-6-sulfonic

acid) (ABTS) solution (150 mg ABTS in 500 ml 0.1 M citric acid, pH 4.35) (Sigma-Aldrich, USA) with 0.03% H_2O_2. The color reaction was stopped after 10 min by adding 50 µl 0.5 M oxalic acid per well. The *Leishmania* loads were determined using the ELISA Reader Tecan (Schoeller Pharma, Czech Republic). The absorbance values of the plates were read at a wavelength of 405 nm with a reference filter of 620 nm. Concentration of *L. major* DNA in the samples was calculated using the curve fitter program KIM-E for Windows (Schoeller Pharma, Czech Republic) by least squares–based linear regression analysis (Kurey et al. 2009, Kobets et al. 2010, Rohoušová et al. 2011).

3. Quantification of parasites using the modified limiting dilution assay

The current semi quantitative technique is based on the limited dilution assay (Titus et al. 1985). We modified the procedure by using only a single pre-selected cell concentration, and parasite count was measured with a Coulter Counter (Beckman Coulter Inc., USA). In comparison with the original limited dilution technique, this modified culture method is less laborious and allows rapid estimation of parasite number. Preparation of cells must be carried out under sterile conditions. During preparation, all samples, which were not immediately worked with, were kept on ice.

Inguinal lymph nodes were disrupted in a glass homogenizer in complete RPMI (containing 5 % of inactivated fetal calf serum (Sigma-Aldrich, USA), 25 mM Hepes (Sigma-Aldrich, USA), 0.0005 % β-mercaptoethanol (Serva, Germany), 63.7 µg/ml penicillin (Sigma-Aldrich, USA), and 100 µg/ml streptomycin (Sigma-Aldrich, USA). The homogenate was passed through the nylon filter. The homogenizer was washed 3 times with 3 ml of sterile PBS after processing each lymph node or spleen. The samples were then centrifuged 8 min at 300×g, 4 ^{o}C using a centrifuge Eppendorf 5810 R (Eppendorf, Germany). Supernatant was removed and the cells were resuspended in 0.5 ml of complete Schneider's medium supplemented with 20 % inactivated fetal calf serum (Sigma-Aldrich, USA), 2 % sterile fresh

human urine, 50 μg/ml gentamicine (Sigma-Aldrich, USA), 63.7 μg/ml penicillin (Sigma-Aldrich, USA), and 100 μg/ml streptomycin (Sigma-Aldrich, USA). To count cells with a Coulter Counter CBC5 (Beckman Coulter Inc., USA), 50 μl of the cell suspension was diluted in 20 ml of PBS. Ekoglobin (Hemax spol. s.r.o., Czech Republic) was added to the diluent prior to counting to lyse red blood cells.

For parasite counting, 0.5 ml of the cell suspension (1×10^5 cells per ml for lymph nodes and 2×10^5 cells per ml for spleens) was cultivated in complete Schneider's medium in 48-well tissue culture plates (Costar, Corning Inc., USA) at 27 ^{o}C in the biological thermostat BT 120 M (Labsystems, Finland) for 3 days. Each sample was prepared in triplicate. After incubation, 100 μl of a mixed sample from each well, containing *Leishmania* parasites released from lymph node or spleen cells, were diluted in 20 ml of PBS and the parasite number was counted with the Coulter Counter (Kobets et al. in preparation).

V. Estimation of cytokine, chemokine and immunoglobulin levels

ELISA procedure was performed similarly as described above in the section "Quantification of *Leishmania* using PCR-ELISA". IFNγ, IL-4, IL-12, and IgE levels in serum were determined using the primary and secondary monoclonal antibodies (IFNγ: R4-6A2, XMG1.2; IL-4: BVD4-1D11, 11B11, BVD6-24G2; IL-12: Red-T, G297-298, C15.6, C17.8; IgE: R35-72, R3592, R35118) and standards from BD Biosciences, USA (recombinant mIFNγ, mIL-4, mIL-12, and purified mIgE: C38-2). The detection limit of the ELISA was determined to be 30 pg/ml for IFNγ, 8 ng/ml for IgE, 16 pg/ml for IL-4 and 15 pg/ml for IL-2 (Kurey et al. 2009, Kobets et al. in preparation).

Specific anti-*P. duboscqi* saliva IgGs were measured by immunoblot and ELISA as previously described (Rohoušová et al., 2005). For ELISA tests, wells were coated with *P. duboscqi* salivary gland homogenate (1/40 gland pair equivalent) in 0.01 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4 °C, sera were diluted 1:200 using 3% horse serum

in PBS with 0.05% Tween 20 (PBS/Tween) (Sigma-Aldrich, USA) and incubated in duplicate for 90 min at 37 °C follwed by incubation with goat anti-mouse IgG-peroxidase conjugate (Sigma-Aldrich, USA) diluted 1:1000 for 45 min at 37 °C. The same ELISA protocol was used to measure specific anti-*L.major* IgG with two minor modifications: wells were coated with crude *L. major* promastigotes (10^6 cell equivalents per well) and sera were diluted 1:400 in PBS/Tween solution. Orthophenylendiamine and H_2O_2 in McIlwein phosphate-citrate buffer (pH 5.5) were used as the substrate solution. The absorbance was measured using the Multiskan RC ELISA reader (Labsystems, USA) at 492 nm wavelengths (Rohoušová et al. 2005, Rohoušová et al. 2011).

Immunoblots were performed on salivary gland homogenate separated by SDS–PAGE on 12.5% gel under non-reducing conditions using the Mini-Protean III apparatus (Bio-Rad, USA). An equivalent of 50 gland pairs was loaded. Separated proteins were electro-transferred onto nitrocellulose membrane by Semi-Phor equipment (Hoefer Scientific Instruments, USA). After transfer, the membrane was cut into strips and incubated with mouse sera diluted 1:100 in Tris buffer (20 mM Tris, 150 mM NaCl, pH 7.6) with 0.05% Tween 20 (Tris/Tween solution) for 1 hour and goat anti-mouse IgG peroxidase-conjugate (heavy chain-specific, Sigma–Aldrich, USA) diluted 1:750 in Tris/Tween solution for 1 hour. The color reaction was developed using substrate solution containing diaminobenzidine and H_2O_2 (Rohoušová et al. 2005, Rohoušová et al. 2011).

Levels of GM-CSF (granulocyte-macrophage colony-stimulating factor), CCL2 (chemokine ligand 2)/MCP-1 (monocyte chemotactic protein-1), CCL3/MIP-1α (macrophage inflammatory protein-1α), CCL4/MIP-1β (macrophage inflammatory protein-1β), CCL5/RANTES (regulated upon activation, normal T-cell expressed, and secreted) and CCL7/MCP-3 (monocyte chemotactic protein-3), in serum were determined using Mouse chemokine 6-plex kit (Bender MedSystems, Austria). Test procedure was performed in 96 well filter plates (Millipore, USA) according to the protocol of Bender MedSystem. The kit

contains two sets of beads of different size internally dyed with different intensities of fluorescent dye. The set of small beads is used for GM-CSF, CCL5/RANTES and CCL4/MIP-1β and set of large beads for CCL3/MIP-1α, CCL2/MCP-1 and CCL7/MCP-3. The beads are coated with antibodies specifically reacting with each of the analytes (chemokines) to be detected in the multiplex system. A biotin secondary antibody mixture binds to the analytes captured by the first antibody. Streptavidin-phycoerythrin binds to the biotin conjugate and emits fluorescent signal. Beads were analyzed on flow cytometer LSR II (BD Biosciences, USA). The limit of detection of each analyte was determined to be for GM-CSF 12.2 pg/ml, CCL2/MCP-1 42 pg/ml, CCL7/MCP-3 1.4 pg/ml, CCL3/MIP-1α 1.8 pg/ml, CCL4/MIP-1β 14.9 pg/ml, CCL5/RANTES 6.1 pg/ml respectively (Kobets et al., in preparation).

VI. Genotyping of F_2 hybrids

DNA was isolated from mouse tails using a standard proteinase procedure (Laird et al. 1991, Kobets at al. 2010). The strain CcS-11 differs from BALB/c at STS-derived segments on eight chromosomes, respectively (Stassen et al. 1996). These differential segments were typed in the F_2 hybrid mice between CcS-11 and BALB/c using 16 microsatellite markers (Research Genetics, USA): D1Mit403, D3Mit45, D7Mit54, D7Mit18, D7Nds1, D7Mit282, D7Mit67, D7Mit259, D8Mit85, D10Mit12, D10Mit46, D12Mit37, D16Mit73, D19Mit51, D19Mit60, and D19Mit46 (all sequences can be found at http://www.informatics.jax.org). The maximal distance between any two markers in the chromosomal segments derived from the strain STS or from the nearest BALB/c derived markers was 19 centimorgan. The PCR genotyping of polymorphic simple sequence length markers with fragment length difference of more than 10 bp was performed using unlabeled primers (D1Mit403, D7Mit18, D7Nds1, D7Mit282, D7Mit67, D7Mit259, D10Mit12, D10Mit46, D12Mit37, D16Mit73, D19Mit51, D19Mit60, and D19Mit46). DNA was amplified in a 20-µl PCR reaction with 0.11 µM of

forward and reverse primer, 0.2 mM concentration of each dNTP, 1.0–3.0 mM $MgCl_2$ (the optimal concentration for the most primers was 1.5 mM), 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 0.5 U of Taq polymerase (Invitrogen, Brazil), and approximately 40 ng of tail DNA. PCR reaction was performed using the I-Cycler (Bio-Rad, USA), according to the following scheme: an initial hot start 3 min at 94 °C, followed by 40 cycles of 94 °C for 30 s for denaturing, 55 °C for 60 s for annealing (except markers D7Mit18 and D7Mit259, for which optimal Ta=57°), 72 °C for 60 s for elongation, and finally 3 min at 72 °C for final extension. Each PCR product was electrophoresed in 2–3% agarose gel with ethidium bromide for 20 min to 1.5 hours at 150 V and analyzed under UV light (Kurey et al. 2009). The PCR genotyping for simple sequence length polymorphic markers with fragment length difference of less than 10 bp (D3Mit45, D7Mit54, and D8Mit85) was performed using [γ-^{32}P]ATP end-labeled primers in 10 μl reaction (Krulová et al. 1997). Each PCR product was denaturated at 99 °C and electrophoresed in 6% acrylamide gels for 1.5–3.5 hours at 50 W. Gels were wrapped in Saran Wrap (Dow Chemicals Co., USA) and exposed to X-ray film FOMA XBU (FOMA, Czech Republic) at -90 ºC for 1.5–24 h or longer time.

VII. Statistical analyses

For statistical estimation of the results, three programs were used: Statistica for Windows 8.0 (StatSoft Inc., USA) (Kobets et al. 2010, Kobets et al., in preparation), NCSS (NCSS, USA) (Kurey et al. 2009, Rohoušová et al. 2011) and PROC GLM statement of SAS 8.2 for Windows (SAS Institute, USA) (Kurey et al. 2009).

Differences in parasite load between BALB/c and STS mouse strains were estimated by the Mann–Whitney *U*-test in Statistica (Kobets et al. 2010).

NCSS and SAS programs were used to detect the linkage of genes that control parasite spread, organ pathology, and immune parameters with microsatellite markers. Genotype, sex, and age were fixed factors and the experiment was random factor in analysis of variance

(ANOVA). We tested individual effects of all markers as well as of all their pairwise combinations using the backward elimination procedure (Tripodis et al. 2001, Kurey et al. 2009).

The differences between CcS/Dem strains in parasite numbers in lymph nodes were evaluated by the analysis of variance (ANOVA) and Newman-Keuls multiple comparison (Kobets et al., in preparation) using Statistica.

Lesion size development was analyzed by general linear models ANOVA and Scheffe's Multiple Comparison Procedure. Other data (parasite load and antibody production) were subjected to non-parametric Wilcoxon tests. For correlation tests we used the non-parametric Spearman Rank Correlation Matrix in NCSS (Rohoušová et al. 2011).

Results

I. *Leishmania* parasite detection and quantification using PCR-ELISA

Tetyana Kobets[1], Jana Badalová[1], Igor Grekov[1], Helena Havelková[1], Milena Svobodová[2], and Marie Lipoldová[1]

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The paper describes an improved and optimized protocol of PCR-ELISA designed for quantitative estimation of *Leishmania* parasite load in organs and tissues of the infected hosts.

- ✓ The primers used in PCR are specific to the conservative region of kinetoplast DNA (kDNA) of *Leishmania*.
- ✓ In contrast to other similar techniques, this method applies two labeled primers: digoxigenin labeled forward primer and biotin labeled reverse primer.
- ✓ Conditions of PCR and all concentrations are carefully optimized that allows achieving extremely high specificity.
- ✓ The sensitivity of PCR-ELISA is 0.3 fg per reaction that approximately corresponds to detection of 0.004 parasites (kDNA is a multiple-copy target, each *Leishmania* organism contains approximately 10 000 kDNA minicircles).
- ✓ PCR-ELISA allows fast analysis of at least 150 samples at the same time.

- ✓ The primer is suitable for quantification of different *Leishmania* species.
- ✓ Results of the novel PCR-ELISA method were validated by comparison with classical calculation of parasites in Giemsa stained lymph node smears.
- ✓ The method has been already successfully applied for two scientific projects, described below (Kurey et al. 2009, Rohoušová et al. 2011).

As the first author, I contributed to the development of the method, performed optimization, measured parasite load in the experimental samples, counted parasites in lymph node smears, performed the statistical analysis and interpretation of the data, and participated in writing of the manuscript.

Leishmania parasite detection and quantification using PCR-ELISA

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This protocol describes an improved and optimized PCR-ELISA method for detection and quantification of *Leishmania* parasites in host tissues. Unlike other DNA-based assays, this method uses digoxigenin- and biotin-labeled primers. This eliminates the need for a separate step of hybridization of the PCR product with labeled probes. The PCR product is detected using sandwich ELISA with antidigoxigenin-detecting antibodies. Primers are complementary to the kinetoplast minicircle conserved region of parasite DNA, allowing the detection of several *Leishmania* species. For measurement of a wide range of parasite concentrations, ±25 cycles were optimal. The sensitivity of this technique is 0.3 fg of parasite DNA per reaction in 40-cycle PCR-ELISA, corresponding to 0.004 parasites. DNA preparation by a standard TRI reagent procedure takes about 4 h. When DNA is prepared, a single person can test a large number of samples (at least 150) in a maximum of 7 h. This method might also be suitable for detecting and quantifying other pathogens, especially for detecting small differences in pathogen numbers.

INTRODUCTION

Infectious diseases are a major health problem in a number of countries. Detection of pathogens confirms a diagnosis, characterizes the host defense and determines the effectiveness of the applied therapy.

The model of *Leishmania* infection in mice reveals complex interactions between the pathogen and the host[1]. Methods of parasite detection include microscopy[2], cell cultures[3–5], immunodetection[6] and DNA-based techniques[7–17]. Culture and microscopy methods are very slow and laborious, particularly when a large number of samples are analyzed. Immunodetection methods based on estimation of anti-*Leishmania* antibody levels do not reflect the exact parasite load because the relation between the presence of antibodies and parasites depends highly on individual responsiveness and the general health condition of the host organism. DNA-based methods of parasite detection and quantification have therefore been developed to overcome these limitations. PCR-ELISA is cheaper than real-time PCR, has high sensitivity and allows the detection of small differences in parasite load[10–12]; however, it is complicated and laborious because it requires a PCR reaction and a subsequent hybridization step. We improved and optimized the PCR-ELISA method[18] for detection and quantification of *Leishmania major* parasites. The new technique uses two labeled primers; therefore, the step of hybridization with labeled probes[10–12] is omitted. By virtue of its incorporated biotin, the PCR product is attached to the streptavidin-coated plate, and can be visualized by peroxidase-linked antidigoxigenin antibody (**Fig. 1**). The primer molecules that were not engaged in the reaction are not detectable by ELISA. Tissues or DNA can be stored at −70 or −20 °C, which allows performing or repeating the measurement at any time.

The present technique can be helpful both in scientific research and in the diagnosis of leishmaniasis. The principle of the method may be used for detection and quantification of other pathogens after selection of appropriate primers.

MATERIALS

REAGENTS

- Parasites (*L. major* LV 561 (MHOM/IL/67/LRC-L137 JERICHO II) promastigotes; see REAGENT SETUP) **! CAUTION** *L. major* is a causal agent of human cutaneous leishmaniasis. Avoid contact of media containing *Leishmania* parasites with open wounds. Always work with gloves in a biological safety cabinet.
- Organs or tissues (lymph nodes obtained from male BALB/cHeA (BALB/c (n = 14)) and STS/A (STS (n = 17)) mouse strains killed 8 weeks after *L. major* infection)[19]
- PBS (see REAGENT SETUP)
- NaCl (Sigma-Aldrich, cat. no. S7653)
- Na_2HPO_4 (Sigma-Aldrich, cat. no. S9390)
- KH_2PO_4 (Sigma-Aldrich, cat. no. P5655)
- KCl (Sigma-Aldrich, cat. no. P9333) **! CAUTION** Avoid contact with skin and eyes and do not breathe dust.
- Lysis buffer (see REAGENT SETUP)
- Tris(hydroxymethyl)-aminomethane (SERVA Electrophoresis, cat. no. 37190) **! CAUTION** Irritating to eyes, respiratory system and skin. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice.
- EDTA-disodium (SERVA Electrophoresis, cat. no. 11280; pH 8.0)
- SDS (Sigma-Aldrich, cat. no. L5750) **! CAUTION** Highly flammable. Harmful when it comes in contact with skin and if swallowed. Irritating to eyes, respiratory system and skin. Wear suitable gloves and eye/face protection, as well as protective clothing. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice.
- Proteinase K (20 mg ml^{-1}; Sigma-Aldrich, cat. no. P6556)
- Isopropanol (PENTA, cat. no. 59300) **! CAUTION** Highly flammable Irritating to eyes. Vapors may cause drowsiness and dizziness. Keep container tightly closed. Keep away from sources of ignition—no smoking.

Avoid contact with skin and eyes. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice.
- Ethanol (PENTA, cat. no. 32294) **! CAUTION** Highly flammable. Keep the container tightly closed. Keep away from sources of ignition—no smoking.
- Chloroform (PENTA, cat. no. 25692) **! CAUTION** It is harmful and there is danger of serious damage to health by prolonged exposure through inhalation and if swallowed. It is irritating to the skin. There is limited evidence of carcinogenic effect. Wear suitable protective clothing and gloves.
- TRI reagent (Molecular Research Center, cat. no. TR 118) **! CAUTION** Contains phenol and thiocyanate. It causes burns. It is poisonous and can be fatal. It is toxic if inhaled, if it comes in contact with skin and if swallowed. It is harmful and there is danger of serious damage to health by prolonged exposure through inhalation and if swallowed. There is a possible risk of irreversible effects. Avoid contact with skin and eyes. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice. Wear suitable gloves and eye/face protection, and also wear protective clothing. In case of accident or if one feels unwell, seek medical advice immediately. Keep away from food, drink and animal food. Avoid release into the environment.
- DNA washing solution (100 mM sodium citrate tribasic hydrate, Sigma-Aldrich, cat. no. 25114); in 10% (vol/vol) ethanol (PENTA, cat. no. 32294)
- NaOH (8 mM; Sigma, cat. no. S8045) **! CAUTION** It causes severe burns. Wear suitable gloves and eye/face protection. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice. In case of accident or if one feels unwell, seek medical advice immediately.
- TE buffer (see REAGENT SETUP)
- Primers complementary to the 120-bp conserved region present in kinetoplast minicircle DNA (**Fig. 1**): digoxigenin-labeled F 5′-ATTTTACAC-CAACCCCCAGTT-3′ and biotin-labeled R 5′-GTGGGGGAGGGGCGT-TCT-3′ (ref. 8) (VBC-GENOMICS Biosciences research)
- PCR buffer without $MgCl_2$ (10×; Invitrogen, cat. no. Y02028 or Sigma-Aldrich, cat. no. P2317-1.5ML)
- $MgCl_2$ (50 mM; Sigma-Aldrich, cat. no. M8266) **! CAUTION** Do not breathe dust. Avoid contact with skin and eyes **▲ CRITICAL** The concentration of $MgCl_2$ must be exact.
- dNTPs (100 mM, deoxynucleotide set; Sigma-Aldrich, cat. no. DNTP100A-1KT)
- Taq DNA polymerase (5 U μl^{-1}) **▲ CRITICAL** The polymerase from Invitrogen, cat. no. 10342-020, is strongly recommended as the most effective and as lacking nonspecific priming under the described conditions (compared with Taq DNA Polymerase from Sigma, cat. no. D4545; DynaZyme II DNA Polymerase from Finnzymes, cat. no. F-503; or Perfect Taq RED from Central European Biosystems, cat. no. CEB001).
- Streptavidin (Sigma-Aldrich, cat. no. S4762)
- Coating buffer (100 mM $NaHCO_3$ (pH 7.0); Sigma-Aldrich, cat. no. S6297)
- ELISA washing solution (0.5% Tween 20 (Sigma-Aldrich, cat. no. P1379) in PBS)
- FCS/PBS (2% (vol/vol) fetal calf serum (Sigma-Aldrich, cat. no. F 2442) in PBS) **▲ CRITICAL** It must be prepared under sterile conditions.
- Antidigoxigenin-detecting antibody Fab fragments (Roche Diagnostic GmbH, cat. no. 11 207 733 910)
- ABTS substrate solution (150 mg ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt); Sigma-Aldrich, cat. no. A1888) in 500 ml of 100 mM citric acid (pH 4.35) (Sigma-Aldrich, cat. no. C7129) **! CAUTION** It is irritating to respiratory system and skin. There is a risk of serious damage to eyes. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice. Wear suitable gloves and eye/face protection, as well as protective clothing **▲ CRITICAL** It must be stored in the dark at −20 °C in 11-ml aliquots in vials; it is not recommended to be refrozen.
- H_2O_2 (30%; PENTA cat. no. 95313) **! CAUTION** Harmful if swallowed. Risk of serious damage to eyes. In case of contact with eyes/skin, rinse immediately with plenty of water and seek medical advice. Wear suitable gloves and eye/face protection, as well as protective clothing. In case of accident or if one feels unwell, seek medical advice immediately.
- Stop solution (500 mM oxalic acid; Sigma-Aldrich, cat. no. 75688) **! CAUTION** Harmful if it comes in contact with skin and if swallowed. Avoid contact with eyes also.

EQUIPMENT
- Biological Safety Cabinet (Telstar BioUltra)
- Centrifuges (Eppendorf 5810 R and Eppendorf MiniSpin, Eppendorf)

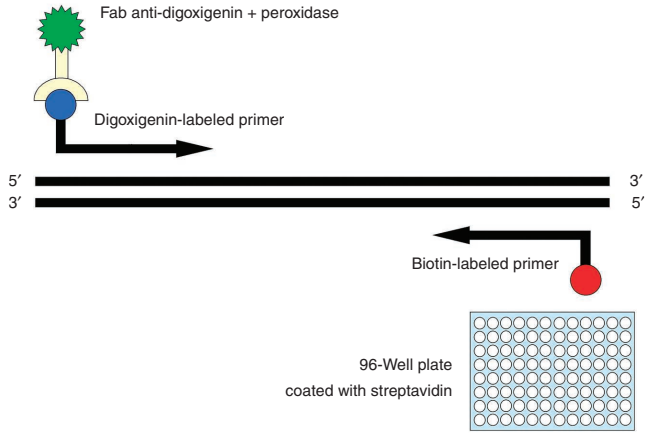

Figure 1 | PCR-ELISA scheme. The method uses two labeled primers for the PCR amplification of target DNA: one is labeled with biotin, the other with digoxigenin. The resulting PCR product (amplicon) with biotin incorporated to one end and dioxigenin to the other is transferred to the microtiter plate coated with streptavidin that binds biotin. The detection of immobilized amplicon is performed by antidigoxigenin antibodies conjugated to peroxidase with final colorimetric development.

- NanoDrop Spectrophotometer (ND-1000, Thermo Scientific)
- DNA Engine DYAD Peltier Thermal Cycler or I-Cycler (Bio-Rad Laboratories)
- ELISA-reader Sunrise (Tecan Group)
- Microtubes (1.5 ml; Axygen, cat. no. MCT-150-C)
- Manual homogenizers (Zymo Research, cat. no H1001-50)
- Pipette tips with filter (Gilson, DF30ST, cat. no. F161933 and DF200ST, cat. no. F161934) for PCR mixture preparation **▲ CRITICAL** Tips with filter should be used to exclude the possibility of contamination.
- 96-well PCR plates (ABgene, cat. no. AB-0600) with cap strips (ABgene, cat. no. AB-0784)
- 96-well ELISA plates (EIA/RIA Clear Flat Bottom Microplate, Corning, cat. no. 3590)
- Curve fitter program KIM-E for Windows (version 5.14, Dan Kittrich, Shoeller Pharma)
- The statistical program Statistica for Windows (version 8.0, StatSoft)

REAGENT SETUP

Cultivate parasites (*L. major* LV 561 (MHOM/IL/67/LRC-L137 JERICHO II) promastigotes) in a diphasic SNB-9 (saline–neopeptone–blood) medium in flat flasks (Nunclon, cat. no. 156340)[20]. Wash stationary-phase promastigotes (7-d-old subculture 2) three times in sterile saline, adjust to 2×10^8 promastigotes per ml in sterile saline and inoculate 50 µl containing 10^7 promastigotes into the mouse tail base[19].

PBS Comprises 140 mM NaCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 2.7 mM KCl. To prepare 1 liter of PBS, dissolve 8.18 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4 and 0.2 g KCl in a small volume of distilled water and adjust volume to 1 liter with distilled water. Solution can be stored at room temperature for up to 1 year.

Lysis buffer Comprises 100 mM tris(hydroxymethyl)-aminomethane, 5 mM EDTA-disodium (pH 8.0), 0.2% SDS and 200 mM NaCl. For preparation of 1 liter of lysis buffer, dissolve 10 ml 20% (wt/vol) SDS, 12.12 g Tris, 10 ml 500 mM EDTA (pH 8.0) and 11.68 g NaCl in a small volume of distilled water and adjust to the final volume of 1 liter with distilled water. To prepare 500 mM EDTA, add 372.3 g of EDTA to 500–700 ml of distilled water, mixing and gradually adding NaOH granules until EDTA is dissolved completely. Adjust pH to 8.0; adjust volume to 1 liter with distilled water. Solution can be stored at room temperature for up to 1 year.

TE buffer Comprises 1 mM EDTA-disodium (pH 8.0) and 10 mM tris(hydroxymethyl)-aminomethane. For preparation of 1 liter of TE buffer, dissolve 1.21 g of Tris and 2 ml of 500 mM EDTA in a small volume of distilled water and adjust volume to 1 liter with distilled water. Solution can be stored at room temperature for up to 1 year.

PROCEDURE

DNA preparation

1| Prepare DNA from parasites or from analyzed tissues. DNA can be prepared in one of two alternative ways: DNA isolation using TRI reagent (based on the protocol of RNA, DNA and protein isolation http://www.mrcgene.com/tri.htm; option A) or through a proteinase procedure[21] (option B).

(A) DNA isolation using TRI reagent ● TIMING ~4 h

(i) Homogenize 50–100 mg of the tissue sample (fresh or frozen) with 1 ml of TRI reagent in a microtube using a manual homogenizer.
▲ **CRITICAL STEP** Sample volume should not exceed 10% of the volume of TRI reagent used for homogenization. Leave the homogenate for 5 min at room temperature (21–23 °C).

(ii) Add 0.2 ml of chloroform per 1 ml of TRI reagent and mix vigorously. Leave the resulting mixture for 2–15 min at room temperature and centrifuge at 12,000*g* for 15 min at 4 °C.

(iii) Remove the aqueous phase overlying the interphase.

(iv) Precipitate DNA from the interphase and organic phase with 0.3 ml of 96% ethanol per 1 ml of TRI reagent used for homogenization; thereafter, mix samples by inversion. Leave the samples at room temperature for 2–3 min and centrifuge at 2,000*g* for 5 min at 4 °C.

(v) Remove the supernatant.

(vi) Wash the pellet twice in 1 ml of DNA washing solution. At each wash, leave the DNA pellet in the DNA washing solution for 30 min at room temperature with periodic mixing by hand and centrifuge at 2,000*g* for 5 min at 4 °C; discard the supernatant.

(vii) Suspend the DNA pellet in 1 ml of 75% ethanol. Set aside for 10–20 min at room temperature with periodic mixing by hand and centrifuge at 2,000*g* for 5 min at 4 °C.

(viii) Remove ethanol and briefly air-dry DNA pellets by keeping tubes open for 5 min at room temperature.

(ix) Dissolve DNA pellets in 0.3 ml of 8 mM NaOH by slowly passing through the pipette tip. Leave DNA samples for about 1 h at room temperature to dissolve.

(x) Centrifuge at 12,000*g* for 10 min to remove insoluble material and transfer the resulting supernatant containing DNA to new tubes.

(xi) Measure DNA concentration using a NanoDrop spectrophotometer.
■ **PAUSE POINT** DNA can be left overnight at 4 °C or stored in a freezer at −20 or −70 °C for years.

(xii) Dilute the DNA samples from tissues to 10 ng μl^{-1}, and parasite DNA (standard) to 4 ng μl^{-1}.
■ **PAUSE POINT** DNA can be left overnight at 4 °C or stored in a freezer at −20 or −70 °C for several years.

(B) Proteinase procedure[21] ● TIMING ~2 d

(i) Add 750 µl of lysis buffer, containing 100 µg ml^{-1} of proteinase K, to the parasite solution (10^8 promastigotes in 500 ml of PBS) or to 50–100 mg of the tissue sample (fresh or frozen). Lyse the samples at 55 °C overnight.

(ii) Centrifuge the samples for 10 min at 3,220*g* at 4 °C to obtain a firm pellet.
▲ **CRITICAL STEP** For different tissues, the time of centrifugation may vary from 10 to 60 min. Nonlysed particles of tissue should be completely removed from the supernatant, because their presence can influence the quality of DNA.

(iii) Transmit the supernatant carefully to the microtube with isopropanol (1:1) for precipitation and centrifuge for 15 min at 3,220*g* at 4 °C.

(iv) Remove the supernatant carefully. Dry the precipitate for ~5 min at room temperature and resuspend in 250 µl of TE buffer. Leave DNA samples overnight at 4 °C to dissolve.

(v) Measure DNA concentration using the NanoDrop spectrophotometer.
■ **PAUSE POINT** DNA can be left overnight at 4 °C or stored in a freezer at −20 or −70 °C.

(vi) Dilute the DNA samples from tissues to 10 ng μl^{-1} and parasite DNA (standard) to 4 ng μl^{-1}.
■ **PAUSE POINT** DNA can be left overnight at 4 °C or stored in a freezer at −20 or −70 °C for a long period of time.

PCR ● TIMING ~2 h 30 min (procedure using 24 cycles)/~3 h 25 min (procedure using 40 cycles)

2| Prepare the reaction mixture. For each DNA sample, use 15 µl of the reaction mixture that contains 0.1 µl of each kinetoplast primer (50 pmol μl^{-1}); 2 µl of 10× PCR buffer without $MgCl_2$; 0.8 µl of 50 mM $MgCl_2$; 0.1 µl of each 100 mM dNTP; 0.4 µl of 5 U μl^{-1} Taq polymerase; and 11.2 µl of bidistilled water.

▲ **CRITICAL STEP** The mixture should be prepared under sterile conditions in a biological safety cabinet.

▲ **CRITICAL STEP** The final concentration of $MgCl_2$ in the reaction must be 2 mM. The primers used are highly sensitive to $MgCl_2$ concentration; subtle deviations from an optimal concentration (2 mM) resulted in either priming of nonspecific DNA synthesis (≥2.5 mM) or inhibition of DNA amplification and product absence (≤1.5 mM).

3| Add 15 µl of the reaction mixture and 5 µl of DNA sample diluted to 10 ng μl^{-1} (50 ng DNA per each reaction) to each well of a 96-well plate. As a standard, use 5 µl of parasite DNA diluted to 4 ng μl^{-1} (20 ng per reaction). As a negative control, add 5 µl of bidistilled water to the reaction instead of DNA.

4| Cover plates tightly with cap strips and run the PCR. The cycling conditions are as follows: 1 cycle at 94 °C for 90 s, followed by 24 cycles at 94 °C for 30 s, 53 °C for 45 s, 72 °C for 60 s and a final extension for 10 min at 72 °C.

ELISA ● TIMING ~20 h

5| Coat 96-well ELISA plates with streptavidin solution (0.5 µg ml^{-1}) in coating buffer at 100 µl per well and leave them overnight at 4 °C. It is possible to coat ELISA plates overnight at 4 °C or at room temperature for 3 h before Step 2 to speed up the procedure.

6| Wash four times with the ELISA washing solution (0.5% Tween 20 in PBS).

7| Dilute PCR samples to 2% concentration using 2% FCS/PBS (usually 2 µl of PCR product and 98 µl of 2% FCS/PBS in each well of the ELISA plate). Each sample should be tested in duplicate.

8| Dilute the standard (the PCR product of 20 ng of *Leishmania* parasite DNA per reaction—see Step 3). To prepare the highest concentration of the standard (2% PCR product in 2% FCS/PBS), dilute 4 µl of PCR reaction product in 196 µl of 2% FCS/PBS in a well of a 96-well ELISA plate. Prepare a series of 10 subsequent dilutions in which each of the subsequent standard samples is 2× diluted: take 100 µl from the first well (containing the most concentrated standard sample), add it to the next well with 100 µl of FCS/PBS, mix the solution by pumping it through the pipette tip, take 100 µl of the mixture from this (second) well and add to the next (third) well (also with 100 µl of FCS/PBS). Proceed until a complete row of 10 dilutions is prepared. Each standard dilution must be prepared in duplicate.

9| Incubate plates at room temperature for 2 h.

10| Wash six times with the ELISA washing solution.

11| Incubate at room temperature with the antidigoxigenin-detecting antibody Fab fragments (100 µl per well of 0.15 U ml^{-1} in 2% FCS/PBS) for 45 min.

12| Wash plates eight times with the ELISA washing solution.

13| Incubate in the dark with 100 µl per well of ABTS substrate solution with H_2O_2 (1 µl of 30% H_2O_2 per 1 ml of ABTS substrate solution) for 5–15 min to develop color reaction.
▲ **CRITICAL STEP** H_2O_2 should be mixed with the ABTS substrate solution immediately before addition.
? TROUBLESHOOTING

14| Stop the color reaction with a stop solution at a concentration of 50 µl per well.

15| Prepare a plate-reading form and a standard curve in the ELISA-reading software according to the manufacturer's instructions. Read the absorbance values of the plates at a wavelength of 405 nm with a reference filter of 620 nm using the ELISA reader. Estimate the concentration of *L. major* DNA in samples using the curve fitter program KIM-E for Windows (or a similar program) using least squares–based linear regression analysis.
? TROUBLESHOOTING

● TIMING

Step 1, DNA preparation
(A) DNA isolation using TRI reagent: ~4 h
(B) Proteinase procedure: ~2 d
Steps 2–4, PCR: ~2 h 30 min (procedure using 24 cycles)/~3 h 25 min (procedure using 40 cycles)
Steps 5–15, ELISA: ~20 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
13, 15	No color developed in standards, nor in the analyzed samples	Decayed H_2O_2	Use fresh H_2O_2
		Decayed ABTS substrate	ABTS substrate solution must be stored in the dark at −20 °C and must not be refrozen. Otherwise prepare a new solution
		Degraded detecting antibody	Ensure that detecting antibody has not expired and is stored at 4 °C. Otherwise use the fresh antibody
		No product in PCR. (It is possible to check the presence of product by running PCR samples on 2% agarose gel)	Ensure that all PCR conditions are proper and concentrations of all reagents are exact. Increase the number of PCR cycles. However, we do not recommend carrying out more than 40 cycles, because at very high cycle numbers the DNA might get degraded
	The color developed both in standards and analyzed samples, but the ELISA-reading software shows that some samples are out of the standard curve and their values are lower than the standard	Some experimental samples contain very low concentration of the parasite DNA. More detailed calibrating curve is necessary to quantitate them	Make more than 10 serial dilutions of the standard used in ELISA and include points of very low concentrations (**Fig. 3a**)
	The color has developed only in the standard samples but not in the analyzed samples	Number of PCR cycles is too small	Increase the number of PCR cycles. However, we do not recommend carrying out more than 40 cycles, because at very high cycle numbers the DNA might get degraded
		Concentration of DNA used in PCR is too low or too high	Try to increase or decrease the amount of total DNA used in PCR. In our experiments, the optimal concentration of the total DNA is 50 ng per reaction, but it can vary depending on the concentration of parasite DNA in total DNA. Using too much total DNA in PCR can inhibit amplification
	The color has developed only in the analyzed samples but not in the standards	Parasite DNA used as standard was degraded	Dilute fresh parasite DNA or isolate new DNA from parasites
	The color is very strong and there are no differences among the analyzed samples	The number of PCR cycles carried out is too high	Carry out a lower number of PCR cycles. However, we do not recommend carrying out less than 15 PCR cycle because at low cycle numbers, the amount of the PCR product is quite small even in samples with high parasite load. Carrying out too few cycles may lead to false negative results
		Concentration of total DNA in the PCR mixture is too high	Use smaller amount of total DNA in the PCR mixture
		The PCR is contaminated with parasite DNA	Prepare the PCR mixture under sterile conditions using pipette tips with filter. Store PCR reagents separated into small aliquots in microtubes. Find out which reagent is contaminated or take new microtubes of all PCR reagents
		Nonspecific products in PCR (check by running on 2% agarose gel)	Ensure that all PCR conditions are proper and concentrations of all reagents are exact. Use only recommended reagents

ANTICIPATED RESULTS

The improved PCR method is able to detect 1.5 fg of parasite DNA (40 cycles of amplification), which corresponds to 0.02 parasites (**Fig. 2**). By introduction of the signal amplification by ELISA, sensitivity was further increased to 0.3 fg of DNA, which approximately corresponds to 0.004 parasites (assuming an average diploid genome mass of 80 fg (ref. 15)). It is much higher

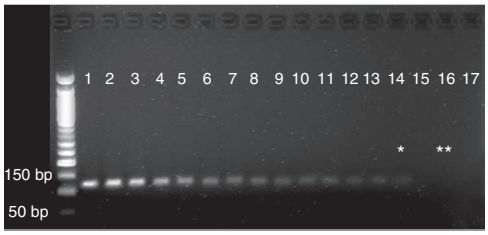

Figure 2 | Sensitivity of detection by PCR. DNA samples were diluted ((1) 5 ng per reaction, (2) 1 ng, (3) 0.5 ng, (4) 0.1 ng, (5) 50 pg, (6) 10 pg, (7) 5 pg, (8) 1 pg, (9) 0.5 pg, (10) 0.1 pg, (11) 20 fg, (12) 5 fg, (13) 3 fg, (14) 1.5 fg, (15) 0.7 fg, (16) 0.3 fg and (17) pure water) and amplified up to 40 cycles. A volume of 20 µl of PCR product was run on 2% agarose gel at 150 V. A 50 bp DNA ladder was used as a marker. *, 1.5 fg per reaction is the minimal DNA concentration at which the amplification product was visible on agarose gel. **, 0.3 fg per reaction is the lowest DNA concentration at which the amplification product was detectable by PCR-ELISA, and it is considered as the technique sensitivity.

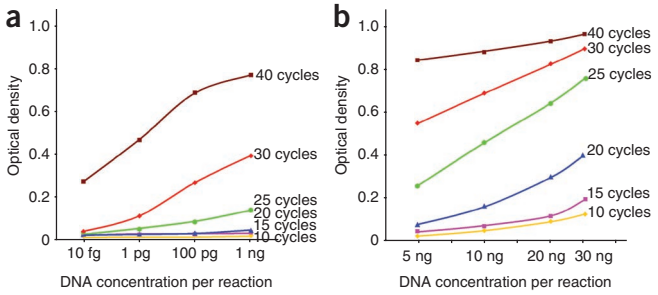

Figure 3 | Standard curves. Comparison of values of optical density obtained with lower (**a**) and higher (**b**) amounts of DNA using different numbers of cycles.

than the sensitivity of culture methods, which are generally lower in sensitivity than PCR methods[22]. It is also more sensitive than microscopy-based methods such as the tissue smear method, which uses a piece of the organ and might give misleading results when parasites are spread unevenly. It has higher or comparable sensitivity compared with other PCR-based techniques[8–17].

We tested the precision of the method by comparing OD values obtained with different amounts of *L. major* DNA using different numbers of cycles of PCR. The results shown in **Figure 3a,b** show a linear relationship between the amount of parasite DNA and the measured OD values over a wide range of DNA concentrations and cycle numbers. The described PCR-ELISA method can be used with 15–40 cycles of PCR. For most purposes, the intermediate number of cycles (around 25) would be optimal. However, the most suitable number of cycles should be selected by the user, taking into account the stage of the disease, susceptibility of the host organism, the organ tested and previous experience.

For analysis of strain differences in parasite loads, we used inguinal lymph nodes from infected male BALB/c and STS mouse strains, which are susceptible and resistant to *L. major*, respectively[23] (**Fig. 4**). Differences in parasite load between the strains were estimated by the Mann–Whitney *U*-test (**Fig. 5**).

The present PCR-ELISA method enabled for the first time the mapping of genetic loci that control parasite numbers in *L. major*-infected mice in a genome-wide search by individually testing 299 F2 hybrids between an intermediate and a susceptible strain[18].

As mentioned above, the primers used in this technique for the detection and quantification of *L. major* parasites are specific to the conserved region present in the kinetoplast minicircle. In addition to *L. major* detection, these primers were previously proven to be suitable for *L. mexicana*, *L. braziliensis*, *L. chagasi*, *L. donovani*, *L. aethiopica* and *L. enterietii*[8], and recently were also successfully tested in our laboratory for the detection and quantification of *L. tropica*. Needless to say, this method, using different primers, can be adapted to measure other parasites, bacteria or viruses.

In summary, the improved PCR-ELISA is an extremely sensitive method that allows simultaneous testing of a large number of samples (at least 150) by a single person and provides results in shorter time.

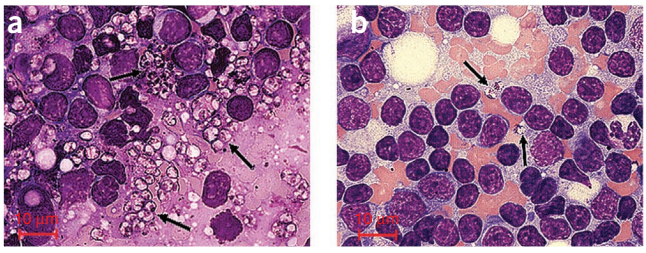

Figure 4 | Giemsa-stained lymph node smears. (**a**) BALB/c mice strain. Arrows show numerous *Leishmania* parasites with a dark nucleus, smaller kinetoplast and light cytoplasm. (**b**) STS mice strain. Arrows show single parasites among lymph node cells. Scale bar (10 µm) was calculated using LAS software (Leica).

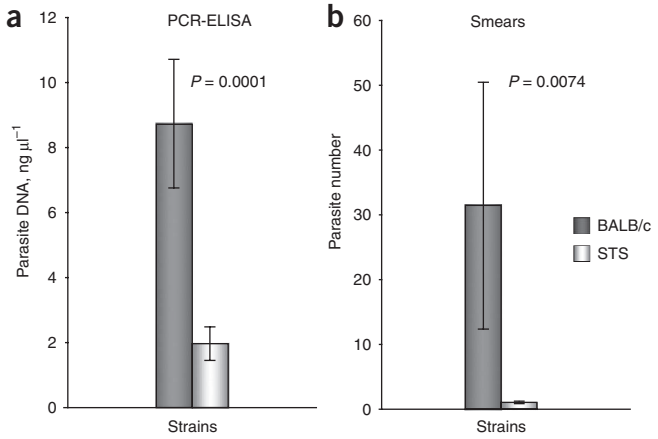

Figure 5 | Parasite load measured with two different techniques. Inguinal lymph nodes from BALB/c ($n=14$) and STS ($n=17$) male mice were used for analysis. (**a**) *Leishmania* DNA concentration measured with the PCR-ELISA technique. DNA was isolated from lymph node homogenates, 50 ng of DNA was amplified per reaction at 24 cycles and 2 µl of PCR product was used for ELISA. (**b**) Parasite count in Giemsa-stained lymph node smears. In each sample, the parasite number was counted as the mean amastigote number from 100 fields observed. Mean and standard error values (± s.e.m.) in BALB/c and STS groups tested by PCR-ELISA, as well as in tissue smears, were obtained by analysis of variance.

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AUTHOR CONTRIBUTIONS T.K., J.B. and M.L. conceived and designed the method; T.K., H.H. and I.G. extended and refined the method; M.S. performed parasitology experiments; and T.K., J.B. and M.L. wrote this paper.

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II. Distinct genetic control of parasite elimination, dissemination, and disease after *Leishmania major* infection

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L. major causes cutaneous form of leishmaniasis in human, but proceeds to more severe visceral pathology in mice. Complex understanding of the host-pathogen interaction is one of the most important questions of medicine. Deep knowledge about mechanisms that contribute to the disease development can help to determine specific therapeutic approaches and make treatment significantly more effective. However, the genetic background of resistance or susceptibility to the disease is often not clear. We used a mouse model to detect genetic loci that are responsible for control of different manifestations during infection with *L. major*. Heterogenic F_2 hybrids between a parental BALB/c strain and CcS-11 recombinant congenic strain showed wide variation in susceptibility to leishmaniasis. The infected mice were tested for several parameters: skin lesion size, splenomegaly (enlargement of the spleen), hepatomegaly (enlargement of the liver), parasite load in inguinal lymph nodes and spleen, levels of IFNγ, IL-4, IL-12, and IgE in blood serum.

- ✓ F_2 hybrids were typed using genetic markers; data about genotypes was applied during statistical analysis to search for loci that control the disease manifestations.

- ✓ Difference in presence of parasites was confirmed by analysis of histological sections.
- ✓ Four novel loci *Lmr20*, *Lmr21*, *Lmr22*, and *Lmr23* (on chromosomes 1, 7, 12, and 16, respectively) were detected.
- ✓ For the previously detected locus *Lmr5* (on the chromosome 10), new functions were described.
- ✓ Presence of *Leishmania* parasites in secondary lymphoid organs is controlled by two loci, *Lmr20* and *Lmr5*. Parasite numbers in spleen and lymph nodes are controlled independently.
- ✓ *Lmr20* on the chromosome 1 is linked to D1Mit403 and it controls parasite load in draining lymph nodes (corrected P=5.54× 10−3). BALB/c allele was associated with higher parasite numbers.
- ✓ *Lmr5* on the chromosome 10 is linked to D10Mit12 and it influences parasite load in spleens and splenomegaly (corrected P=5.39×10−3 and 6.98×10−4, respectively). Higher parasite numbers and a more severe splenomegaly was associated with BALB/c allele.
- ✓ There was also a pronounced sex effect (P=10−9) on parasite numbers in draining lymph nodes and spleen.
- ✓ Besides parasite load in lymph nodes, *Lmr 20* controls serum IgE, serum IFNγ (in interaction with *Lmr23*).
- ✓ In addition to parasite load in spleen, *Lmr5* controls lesion size, splenomegaly, serum IgE, serum IFNγ (in interaction with *Lmr21*), serum IL-4 (in interaction with *Lmr22*).
- ✓ *Lmr21* (chromosome 7) controls lesion, serum IFNγ (in interact with *Lmr5*)**.**

- ✓ *Lmr22* (chromosome 12) controls serum IL-4 (in interaction with *Lmr5*)**.**

- ✓ *Lmr23* (chromosome 16) controls serum IFNγ (in interaction with *Lmr20*).

As the second author, I contributed to isolation of organs from the infected mice, performed measurement of parasite load in spleen and lymph nodes using PCR-ELISA, analyzed H&E stained histological sections, contributed to data interpretation and preparation of the manuscript.

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ORIGINAL PAPER

Distinct genetic control of parasite elimination, dissemination, and disease after *Leishmania major* infection

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Abstract Elimination of pathogens is the basis of host resistance to infections; however, relationship between persisting pathogens and disease has not been clarified. *Leishmania major* infection in mice is an important model of host–pathogen relationship. Infected BALB/c mice exhibit high parasite numbers in lymph nodes and spleens, and a chronic disease with skin lesions, splenomegaly, and hepatomegaly, increased serum IgE levels and cytokine imbalance. Although numerous gene loci affecting these disease symptoms have been reported, genes controlling parasites' elimination or dissemination have never been mapped. We therefore compared genetics of the clinical and immunologic symptomatology with parasite load in (BALB/c×CcS-11) F_2 hybrids and mapped five loci, two of which control parasite elimination or dissemination. *Lmr5* influences parasite loads in spleens (and skin lesions, splenomegaly, and serum IgE, IL-4, and IFNγ levels), and *Lmr20* determines parasite numbers in draining lymph nodes (and serum levels of IgE and IFNγ), but no skin or visceral pathology. Three additional loci do not affect parasite numbers but influence significantly the disease phenotype—*Lmr21*: skin lesions and IFNγ levels, *Lmr22*: IL-4 levels, *Lmr23:* IFNγ levels, indicating that development of *L. major*-caused disease includes critical regulations additional to control of parasite spread.

Keywords *Leishmania major* · Mouse · Genetics of parasite elimination · Susceptibility to leishmaniasis · Functional heterogeneity

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Parasite infection induces in the host both the responses that limit parasite growth and multiplication and responses that are either neutral or can lead to damage of host's tissues. Interaction between the infecting parasite and the host's immune system is especially important for understanding of pathogenesis of diseases caused by chronic infections.

One of the important unresolved questions in pathogenesis of chronic infections is the relationship between the pathogen load and clinical symptoms. Whereas early elimination of infectious agents generally precludes development of chronic disease, the relationship between load and spread of persisting pathogens and manifestations and course of ensuing disease has not been clarified. Although the number of studies addressing this problem is limited, they provided examples of unexpected genetic and functional complexity in infections with various pathogens such as *Borrelia burgdorferi* (Ma et al. 1998), *Toxoplasma*

gondii (Johnson et al. 2002), and *Trypanosoma cruzi* (Marinho et al. 2004). This involved strain differences in both the relationship between pathogen load and intensity of symptoms of disease and in dissemination to different organs. However, the relationship between genetic control of parasite load and parasite-induced pathology has never been analyzed simultaneously in segregating populations (Råberg et al. 2007).

Leishmania infection in mice has served as a paradigm of interaction of pathogen and host. *Leishmania* parasites are transmitted to vertebrate hosts by infected phlebotomine sandflies and infect professional phagocytes (macrophages, monocytes, and neutrophils) as well as dendritic cells and fibroblasts. The major host cell is macrophage, in which parasites multiply, eventually rupturing the cell and spreading to uninfected cells. They affect skin, mucous membranes, lymph nodes, spleen, bone marrow, liver, lungs, and, in rare cases, even the brain. The expansion of the parasites is determined by multiple factors, including both parasite characteristics (reviewed in McMahon-Pratt and Alexander 2004) and host genetics (reviewed in Lipoldová and Demant 2006). *Leishmania* parasites can evade the first-line defense systems, inhibit several functions of host's immune cells (Olivier et al. 2005), and prime the development of an immune state favorable for the parasite. In the mouse model, distinct clinical manifestation of cutaneous and visceral leishmaniasis observed in humans can be reproduced in some mouse strains. Mice from several strains (BALB/c, SWR/J, P/J) develop severe systemic disease affecting skin and visceral organs (Sacks and Noben-Trauth 2002), whereas mice of the majority of inbred strains (e.g., C3H, CBA, C57BL/6, B10.D2, 129/Sv, and STS/A) after infection with *L. major* develop no pathological changes or only small spontaneously healing cutaneous lesions restricted to the infection site. The wide spectrum of clinical symptoms and immunological reactions in susceptible strains provides an opportunity to define control of individual components of disease phenotype. However, there are no data about the relationship of parasite load to manifestations of disease.

As the non-healer phenotype of BALB/c mice is controlled by multiple genes, we used a special tool for genetic analysis of multigenic traits, the recombinant congenic (RC) strains. Each of the 20 CcS/Dem RC mouse strains carries a different random subset of 12.5% genes of the resistant donor strain STS/A (STS) on the background of the susceptible strain BALB/cHeA (BALB/c). As a consequence, the individual STS genes contributing to the resistance to infection *L. major* were distributed among different RC strains. Previously, we studied three CcS/Dem strains: the most resistant strain CcS-5, the strain CcS-20 that exhibits intermediate susceptibility, and the susceptible strain CcS-16. This resulted in mapping of 17 loci, *Lmr3–Lmr19* (*Leishmania major* response 3–19), which are associated with 13 different combinations of pathological symptoms and immunological reactions (Lipoldová et al. 2000; Badalová et al. 2002; Vladimirov et al. 2003; Havelková et al. 2006).

In the present study, in addition to the disease symptoms and immunological parameters, we also measured load of parasites in draining lymph nodes and spleen. This allowed us to analyze for the first time the genetic relationship between the presence of parasites and manifestations of disease. We mapped genes controlling these parameters in the strain CcS-11, which after infection presents smaller skin lesions, lower splenomegaly and hepatomegaly, fewer parasites in spleen, and lower concentrations of IgE and IL-4 in serum than the susceptible strain BALB/c (Lipoldová et al. 2002 and this paper). We show here that the degree of elimination of parasites is genetically controlled and that this control is organ specific. Different genes control parasite load predominantly in lymph nodes or in spleen. Moreover, we show that additional genes that do not affect parasites' presence in these two organs also significantly affect the course of disease.

Materials and methods

Mice Males and females of strains BALB/c and CcS-11 were 8 to 24 weeks old at the time of infection. When used for these experiments, strain CcS-11 was in more than 38 generations of inbreeding. The parts of its genome inherited from the BALB/c or STS parents were defined (Stassen et al. 1996). Two hundred and ninety-nine F_2 hybrids between CcS-11 and BALB/c (age 9 to 13 weeks at the time of infection, mean age 11 weeks, median 12 weeks) were produced at the Institute of Molecular Genetics. They comprised 155 females and 144 males. During the experiment, male and female mice were placed into separate rooms and males were caged individually. They were tested in three successive experimental groups. The research had complied with all relevant European Union guidelines for work with animals.

Parasites Leishmania major LV 561 (MHOM/IL/67/LRC-L137 JERICHO II) was maintained in rump lesions of BALB/c females. Amastigotes were transformed to promastigotes using SNB-9 (Diamond and Herman 1954). Promastigotes (10^7) from 6-day-old subculture 2 were inoculated in 50 μl sterile saline s.c. into mouse rump.

Disease phenotype The size of the primary skin lesions was measured weekly using a Vernier caliper gauge. The mice were killed 8 weeks after infection. Blood, spleen, liver,

and inguinal lymph nodes were collected for further analysis.

Quantification of parasite load in spleens and inguinal lymph nodes by PCR–ELISA Total DNA from spleens and inguinal lymph nodes was prepared using a standard proteinase procedure. To detect *Leishmania* DNA by PCR, two primers (digoxigenin-labeled F 5′-ATT TTA CAC CAA CCC CCA GTT-3′ and biotin-labeled R 5′-GTG GGG AGG GGG CGT TCT-3′; Rodgers et al. 1990; VBC-GENOMICS Biosciences Research, Vienna, Austria) were used for amplification of the 120-bp conservative region of the minicircle kinetoplast of *Leishmania* parasites. Briefly, 50 ng of extracted DNA with 5 pmol of each kinetoplast primer, 0.5 mM concentration of each dNTP, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), and 2 U of Taq polymerase (Invitrogen Brazil Ltda., São Paulo, Brazil) was amplified in a total volume of PCR reaction of 20 μl. As a positive control, we used 20 ng of *L. major* DNA per reaction (0.3 fg of *L. major* DNA approximately corresponds to one parasite). After an initial hot start at 94°C for 90 s, the conditions used for amplification were: 94°C for 30 s, 53°C for 45 s, 72°C for 60 s (26 cycles for DNA from lymph nodes and 33 cycles for DNA from spleens), followed by the final extension 72°C for 10 min, using the I-Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Concentration of the amplified 120-bp region of *Leishmania* DNA in PCR product was determined using the modified ELISA protocol (Pharmingen, San Diego, CA, USA). Plates were coated with 100 μl per well streptavidin solution (0.5 μg/ml in 0.1 M $NaHCO_3$ pH 7.0) at 4°C overnight. PCR samples were diluted to 2% concentration in 2% FCS/PBS. The positive PCR control was used as a standard for ELISA. Plates were incubated at room temperature for 2 h and then incubated at room temperature for 45 min with the anti-digoxigenin detecting antibody, Fab fragments (Roche Diagnostic GmbH, Mannheim, Germany) 0.15 U/ml, diluted in 2% FCS/PBS. As a substrate for color reaction development, we used 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) solution (150 mg ABTS in 500 ml 0.1 M citric acid, pH 4.35) (Sigma-Aldrich Co., St. Louis, MO, USA) with 0.03% H_2O_2. The color reaction was stopped after 10 min by adding 50 μl 0.5 M oxalic acid per well. The *Leishmania* loads were determined using the ELISA Reader Tecan and the curve fitter program KIM-E (Schoeller Pharma, Prague, Czech Republic) using least squares-based linear regression analysis.

Cytokine and IgE levels IFNγ, IL-4, IL-12, and IgE levels in serum were determined using the primary and secondary monoclonal antibodies (IFNγ=R4-6A2, XMG1.2; IL-4=BVD4-1D11, BVD6-24G2; IL-12=Red-T and G297-298; IgE=R35-72, R3592) and standards from Pharmingen, San Diego, CA (recombinant mIFNγ, mIL-4, mIL-12 p70 heterodimer, and purified mIgE=C38-2). The ELISA measurements of IFNγ, IL-4, IL-12, and IgE levels were performed as described in the previous section.

Genotyping of F_2 mice DNA was isolated from tails using a standard proteinase procedure. The strain CcS-11 differs from BALB/c at STS-derived segments on eight chromosomes, respectively (Stassen et al. 1996). These differential segments were typed in the F_2 hybrid mice between CcS-11 and BALB/c using 16 microsatellite markers (Research Genetics, Huntsville, FL, USA): D1Mit403, D3Mit45, D7Mit54, D7Mit18, D7Nds1, D7Mit282, D7Mit67, D7Mit259, D8Mit85, D10Mit12, D10Mit46, D12Mit37, D16Mit73, D19Mit51, D19Mit60, and D19Mit46. The maximal distance between any two markers in the chromosomal segments derived from the strain STS or from the nearest BALB/c derived markers was 19 cM.

The PCR genotyping of polymorphic simple sequence length markers with fragment length difference of more than 10 bp was performed using unlabeled primers (D1Mit403, D7Mit18, D7Nds1, D7Mit282, D7Mit67, D7Mit259, D10Mit12, D10Mit46, D12Mit37, D16Mit73, D19Mit51, D19Mit60, and D19Mit46). DNA was amplified in a 20-μl PCR reaction with 0.11 μM of forward and reverse primer, 0.2 mM concentration of each dNTP, 1.0–3.0 mM $MgCl_2$ (the optimal concentration for the most primers was 1.5 mM), 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 0.5 U of Taq polymerase (GIBCO, Grand Island, NY, USA), and approximately 40 ng of tail DNA. PCR reaction was performed using the I-Cycler (Bio-Rad Laboratories), according to the following scheme: an initial hot start 3 min at 94°C, followed by 40 cycles of 94°C for 30 s for denaturing, 55° for 60 s for annealing (except markers D7Mit18 and D7Mit259, for which optimal T_a=57°), 72° for 60 s for elongation, and finally 3 min at 72° for final extension. Each PCR product was mixed with 5 μl of loading buffer and electrophoresed in 2–3% agarose gel for 20 min to 1.5 h at 150 V. The PCR genotyping for simple sequence length polymorphic markers with fragment length difference of less than 10 bp (D3Mit45, D7Mit54, and D8Mit85) was performed using [γ-^{32}P]ATP end-labeled primers as described elsewhere (Krulová et al. 1997).

Statistical analysis The linkage of genes that control parasite spread, organ pathology, and immune parameters with microsatellite markers was examined with analysis of variance (NCSS, Kaysville, UT, USA) and PROC GLM statement of SAS 8.2 for Windows (SAS Institute, Gary, NC, USA). Genotype, sex, and age were fixed factors and the experiment was random factor. In order to obtain

normal distribution, the obtained values of dependent variables were transformed as shown in the legends of the tables. We tested individual effects of all markers as well as of all their pairwise combinations using the backward elimination procedure (Tripodis et al. 2001). The phenotypes of compound genotypes that significantly deviated from simple additive effects of the alleles indicated interactions. Markers and interactions with P <0.05 were combined in a single comparison.

The time course of skin lesion development was evaluated on the basis of weekly measurements of lesion size in each mouse in weeks 4 to 8 after infection. Mixed model of analysis of variance (Proc Mixed, SAS) with marker as the fixed variable, the week of observation as the covariate, and mouse as random variable have been used to evaluate the linkage.

The percentage of variance explained by an independent variable, the partial R^2, was computed in the standard way by subtracting the regression sums of squares of the model without the variable of interest (SS(β_1, β_2, β_3, β_4/β_0)) from the regression sums of squares of the full model (SS(β_1, β_2, β_3, β_4, β_5/β_0)); this difference divided by the residual sums of squares (RSS(β_1, β_2, β_3, β_4, β_5/β_0)) algebraically expressed as

$$\frac{(\mathrm{SS}(\beta_1,\beta_2,\beta_3,\beta_4/\beta_0)) - (\mathrm{SS}(\beta_1,\beta_2,\beta_3,\beta_4,\beta_5/\beta_0))}{\mathrm{RSS}(\beta_1,\beta_2,\beta_3,\beta_4,\beta_5/\beta_0)}$$

indicated the contribution of the independent variable.

To obtain genome-wide significance values (corrected P), the observed P values (α_T) were adjusted according to Lander and Schork (1994) using the formula:

$$\alpha_T* \approx [C + 2\rho G h(T)]\alpha_T$$

where G=1.75 M (the length of the segregating part of the genome=12.5% of 14 M), C=8 (number of chromosomes segregating in cross between CcS-11 and BALB/c, respectively); ρ=1.5 for F_2 hybrids; $h(T)$=the observed statistic (F ratio). Direct comparisons of this method with permutation analyses in establishing genome-wide significance thresholds found them comparable (Singer et al. 2005).

Power computation for detection of sex difference in marker effects on parasite load were carried forth. Across genotypes (D1Mit403, D10Mit12), the sample size was balanced between males (n=144) and females (n=144) for D1Mit403 and males (n=143) and females (n=146) for D10Mit12. The marker frequencies for each genotype ranged for CC from 59 to 65, for CS from 149 to 157, and for SS from 66 to 79. Under these sample sizes, we were able to detect on the log scale at 0.80 power and α= 0.05 (two-sided) effect sizes for sex by marker interaction in ranges from 0.75 to 0.86 standard deviation units or larger, depending upon the specific differential effect.

Results

Differences in organ pathology and immune response between strains CcS-11 and BALB/c The parasite load, disease symptoms, and immunological parameters in BALB/c and CcS-11 mice are given in Table 1. BALB/c mice develop larger skin lesions during the course of infection (weeks 5, 6, 7, and 8) and larger splenomegaly and hepatomegaly compared to the strain CcS-11, with no significant sex difference in size of skin lesions, splenomegaly, and hepatomegaly (data not shown). Larger numbers of parasites in spleen were also observed in strain BALB/c, but there were no significant differences in parasite load in draining lymph nodes. Parasite numbers in spleens of strain CcS-11 and in draining lymph nodes of both BALB/c and CcS-11 were significantly higher in males (Table 1, Fig. 1). Serum IgE and IL-4 levels were higher in strain BALB/c than in strain CcS-11, but there were no strain differences in serum concentrations of IFNγ and IL-12. Serum IgE and cytokine levels were not influenced by sex (Table 1).

Loci that influence parasite burden, organ pathology, and immunological parameters in the strain CcS-11 The summary of the detected loci and their phenotypic effects is given in Table 2. Linkage analysis of F_2 hybrids between BALB/c and CcS-11 indicated four novel loci *Lmr20*, *Lmr21*, *Lmr22*, and *Lmr23* on chromosomes 1, 7, 12, and 16, respectively. For the previously detected locus *Lmr5* on chromosome 10 (Lipoldová et al. 2000), we detected an additional effect on parasite load in spleen and on IL-4 level in serum in interaction with *Lmr22*. These data are given in Tables 3, 4, and 5 (single gene effects) and Tables 6, 7, and 8 (gene interactions).

These tables describing detected linkages give besides the statistical significance of linkage also two other quantitative characteristics (Lander and Botstein 1989) that describe the size of their effect: (a) a magnitude of effect of each locus that describes how large the phenotypic difference is (e.g., in IgE level) between mice with different alleles at this locus and (b) the explained variance (% of variance) that is the proportion of phenotypic variation in the tested population that is caused by the effects of these alleles. The first parameter in our data varies between 1.4 and 2.5 (i.e., difference between the extreme phenotypes caused by alleles at a locus is between 40% and 150%) and the percent of explained variance 1–14. These value ranges are common for majority of quantitative trait loci (QTLs) in the mouse (Flint et al. 2005).

Parasite presence in secondary immune organs is controlled by two loci, *Lmr20* and *Lmr5* (Table 3). *Lmr20* on chromosome 1 is linked to D1Mit403 and it controls

Table 1 Comparison of immunopathological parameters in *L. major* infected mice of the strains BALB/c and CcS-11

Phenotype	Strain				*P* value
	BALB/c		CcS-11		Strain difference
Lesion size 5 weeks (mm^2)		**60.79±6.33** (n=19)		**29.55±6.33** (n=19)	1.34×10^{-3}
Lesion size 6 weeks (mm^2)		**91.67±7.78** (n=19)		**41.33±7.78** (n=19)	5.88×10^{-5}
Lesion size 7 weeks (mm^2)		**107.42±9.52** (n=19)		**46.54±9.52** (n=19)	6.91×10^{-5}
Lesion size 8 weeks (mm^2)		**129.31±11.73** (n=19)		**55.05±11.73** (n=19)	7.86×10^{-5}
Splenomegaly (spleen-to-body weight ratio×1,000)		**17.84±1.13** (n=19)		**12.26±1.13** (n=19)	1.51×10^{-3}
Hepatomegaly (liver-to-body weight ratio×1,000)		**68.06±1.55** (n=19)		**60.32±1.55** (n=19)	2.75×10^{-3}
IgE in serum (μg/ml)	**10.22**	3.54±0.29 (n=16)	**3.97**	1.62±0.28 (n=18)	6.79×10^{-5}
IFNγ in serum (ng/ml)	**5.57**	1.31±0.05 (n=18)	**4.45**	1.22±0.06 (n=14)	0.257
IL-12 in serum (ng/ml)	**2.83**	1.04±0.20 (n=16)	**1.74**	0.55±0.20 (n=15)	0.119
IL-4 in serum (ng/ml)	**4.65**	1.61±0.23 (n=12)	**2.33**	0.83±0.26 (n=9)	3.75×10^{-2}
Parasites in draining lymph nodes, total group (arbitrary units)	**1.61**	5.08±0.20 (n=17)	**2.30**	5.44±0.19 (n=19)	0.211
Parasites in draining lymph nodes, females	**0.57**	4.04±0.23[a] (n=9)	**0.46**	3.83±0.22[b] (n=10)	0.643
Parasites in draining lymph nodes, males	**9.28**	6.83±0.37[a] (n=8)	**8.54**	6.75±0.35[b] (n=9)	0.931
Parasites in spleen, total group (arbitrary units)	**1.58**	5.06±0.29 (n=18)	**0.31**	3.43±0.28 (n=19)	4.02×10^{-4}
Parasites in spleen, females	**1.10**	4.70±0.45[c] (n=8)	**0.18**	2.89±0.40[d] (n=10)	8.36×10^{-3}
Parasites in spleen, males	**3.08**	5.73±0.34[c] (n=10)	**0.58**	4.06±0.36[d] (n=9)	6.01×10^{-3}

Means and standard errors of means and *P* values were computed by analysis of variance. Lesion size (mm^2), splenomegaly and hepatomegaly (spleen and liver-to-body weight ratio×1,000, respectively) exhibited normal distribution without transformation. The following transformations were used to obtain normal distribution: IgE levels in serum: IgE (μg/ml)—natural logarithm of value to the power of 1.5; IFNγ (ng/ml)—natural logarithm of the value to the power of 0.5; IL-4 (ng/ml)—natural logarithm to the power of 1.1; IL-12 (ng/ml)—natural logarithm; parasite load detected as parasite DNA content (arbitrary units) in spleen and in lymph nodes—natural logarithm of (value×100). Number of tested mice is shown in brackets. The numbers in bold give the average non-transformed values.

[a] Sex difference in BALB/c, $P=9.26\times10^{-5}$

[b] Sex difference in CcS-11, $P=1.14\times10^{-5}$

[c] Sex difference in BALB/c, $P=0.133$

[d] Sex difference in CcS-11, $P=8.93\times10^{-3}$

parasite load in draining lymph nodes (corrected $P=5.54\times10^{-3}$). Its BALB/c allele is associated with higher parasite numbers. The effects of *Lmr20* account for 4.25% of variance in parasite load in the lymph nodes. In addition, there was a pronounced sex effect ($P=10^{-9}$) on parasite numbers in draining lymph nodes and spleen. However, there was no interaction between sex and the marker and no sufficient power to establish whether the effects of *Lmr20* are sex specific. This qualification concerns the effects of the individual *Lmr* loci; the sex difference within the two strains is highly significant (Table 1).

Lmr5 on chromosome 10 is linked to D10Mit12 and it influences parasite load in spleens and splenomegaly (corrected $P=5.39\times10^{-3}$ and 6.98×10^{-4}, respectively). The effects of *Lmr5* account for 4.54% of variance in parasite number in spleens and 11.60% of variance in splenomegaly. Higher parasite numbers and a more severe splenomegaly are associated with BALB/c allele. Separate analysis of females and males revealed that *Lmr5* determines parasite numbers in spleen and splenomegaly in females (corrected $P=5.76\times10^{-3}$ and 5.57×10^{-4}, respectively). It accounts in females for 11.74% and 14.47% of variance in parasite numbers and splenomegaly, respectively, whereas differences in parasite numbers in spleens and splenomegaly in males were not significant (Table 3). However, absence of interaction between sex and marker and power computations indicated that the present experiments did not have sufficient power to establish whether *Lmr5* has a sex-specific effect. The present data are compatible with additive effects of sex and genotype at *Lmr5*.

Skin lesions are controlled by two loci: *Lmr5* and *Lmr21* (Table 4 and Fig. 2). *Lmr5* on chromosome 10 is linked to D10Mit12 and D10Mit46 (corrected $P=5.27\times10^{-5}$ and 4.83×10^{-5}, respectively). *Lmr21* on chromosome 7 is linked to D7Mit67 and D7Mit282 (lesion size on week 8; corrected $P=0.0116$ and 0.0972). BALB/c allele of both *Lmr5* and *Lmr21* is associated with larger lesions. The impact of *Lmr5* and *Lmr21* on phenotype is higher in

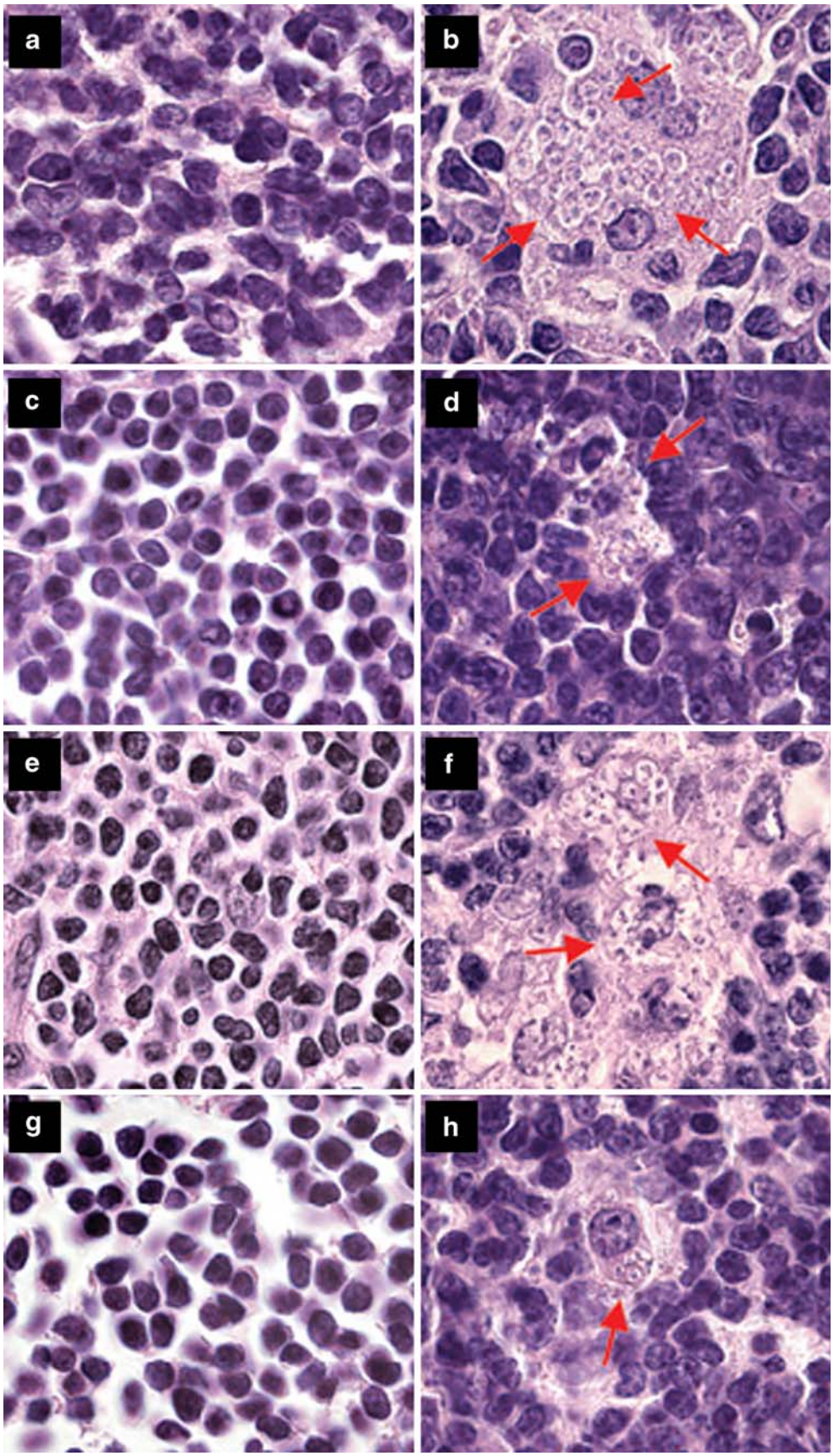

Fig. 1 Parasites in hematoxylin–eosin-stained inguinal lymph node sections of female and male mice. **a** Uninfected BALB/c male, **b** *L. major* infected BALB/c male, **c** uninfected BALB/c female, **d** *L. major* infected BALB/c female, **e** uninfected CcS-11 male, **f** *L. major* infected CcS-11 male, **g** uninfected CcS-11 female, **h** *L. major* infected CcS-11 female. *Arrows* show groups of *Leishmania* parasites (with a dark nucleus, smaller kinetoplast, and light cytoplasm) among normal lymph node cells (**b**, **d**, **f**) and parasites inside a macrophage (**h**)

Table 2 Summary of *Lmr* loci that control response to *L. major* in the strain CcS-11

Locus	Chr.	Marker (position in cM)	Trait controlled
Lmr20	1	D1Mit403 (100)	Parasite load in draining LNs, serum IgE, serum IFNγ (in interaction with *Lmr23*)
Lmr21	7	D7Mit67 (63.5)	Lesion development, serum IFNγ (in interaction with *Lmr5*)
Lmr5	10	D10Mit12 (56); D10Mit46 (63)	Lesion size, parasite load in spleen, splenomegaly, serum IgE, serum IFNγ (in interaction with *Lmr21*), serum IL-4 (in interaction with *Lmr22*)
Lmr22	12	D12Mit37 (1)	Serum IL-4 (in interaction with *Lmr5*)
Lmr23	16	D16Mit73 (8.6)	Serum IFNγ (in interaction with *Lmr20*)

Only the QTLs with corrected $P<0.05$ (genome-wide significance) are shown.

females than in males. In females, *Lmr5* on chromosome 10 is linked with D10Mit12 and D10Mit46 (corrected $P=2.67\times10^{-4}$ and 7.28×10^{-4}, respectively), and *Lmr21* on chromosome 7 is linked with D7Mit67 (corrected $P=0.046$). In males, we did not detect *Lmr5* or *Lmr21.*

Components of the systemic immune response: Of the four tested components (serum levels of IgE, IFNγ, IL-4, and IL-12) we did not detect any linkage of IL-12 levels. The linkages of the other three components involve five loci, *Lmr20, Lmr21, Lmr5, Lmr22* and *Lmr23* (Table 5 single gene effects and Tables 6, 7, and 8 gene interactions). Serum IgE concentrations are influenced by *Lmr20* and *Lmr5*. *Lmr20* on chromosome 1 is linked to D1Mit403 (corrected $P=3.07\times10^{-3}$, 6.09% of variance) and its BALB/c allele is associated with higher serum levels of IgE. Influence of this locus on IgE level is observed only in females (corrected $P=1.81\times10^{-3}$, 11.76% of variance). *Lmr5* on chromosome 10 is linked to D10Mit12 (corrected P value$=3.02\times10^{-2}$, 4.70% of variance). When analyzed separately, females and males exhibit allelic differences of the same size and direction, but do not reach significant P values due to insufficient number of mice in the separate sexes.

Serum IFNγ concentrations are influenced by two inter-locus interactions. *Lmr20* marked by D1Mit403 interacts with *Lmr23* linked to D16Mit73 (Table 6). The highest level of IFNγ is observed in STS homozygotes in both loci (corrected $P=2.01\times10^{-2}$). This interaction accounts for 6.93% of variance.

Serum IFNγ level is also controlled by the interaction of *Lmr21* (linked to D7Mit67) with *Lmr5* (linked to D10Mit12) (corrected $P=1.95\times10^{-2}$, 7.18% of variance). The lowest IFNγ levels are observed either in STS homozygotes at both loci or in BALB/c homozygotes in both loci (Table 7). A separate statistical evaluation of these interactions in females and males was not possible due to the low numbers of mice in some genotypes, but the interaction effects were similar in both sexes (data not shown).

Serum IL-4 concentration is influenced by one inter-locus interaction. *Lmr22* marked by D12Mit37 interacts with *Lmr5* linked to D10Mit12. The highest and lowest level of IL-4 is observed in BALB/c and STS homozygotes, respectively, at both loci (corr. $P=2.69\times10^{-2}$). This interaction accounts for 7.71% of variance (Table 8).

Sex differences in genetic control of lesion size, splenomegaly, and serum level of IgE were revealed when analyzing the male and female F_2 hybrids separately. Influence of sex was high for skin lesions during the whole course of infection ($P<1.68\times10^{-5}$) and IgE level in serum ($P=1.41\times10^{-2}$). The impact of the *Lmr* loci controlling these parameters appears to be stronger in females than in males. However, this influence of sex was independent of the segregating genes, as there was no significant interaction between sex and any of the tested markers.

Discussion

There is extensive literature about genetic control of *L. major*-induced disease (Demant et al. 1996; Beebe et al. 1997; Roberts et al. 1997, 1999; Lipoldová et al. 2000; Badalová et al. 2002; Vladimirov et al. 2003; Havelková et al. 2006), but until now there are no reports on the genetics of parasite elimination. However, the genetics of host–pathogen interactions has two important parts: genetics of infection that describes genes that participate in the control of the spread of the infectious agent within the host and genetics of disease development that analyzes the immune and pathological responses of the host. We used genetic dissection to elucidate the functional relationship between the two components of the disease. This study represents the first genome-wide search for loci controlling parasite elimination and dissemination after *L. major* infection. Our results show that these processes correlate only partly.

Controlling loci and their function We have detected five loci that in the strain CcS-11 control host–parasite interaction (Table 2, Fig. 3). We have found that parasite

Table 3 Loci that control parasite load in draining lymph nodes and spleens and splenomegaly in *L. major* infected F_2 hybrids between CcS-11 and BALB/c

Phenotype	Locus	Group	Marker	Genotype						*P* value	Corrected *P* value	Percent of explained variance
				CC		CS		SS				
Parasites in draining lymph nodes (arbitrary units)	*Lmr20*	Both sexes	D1Mit403		**7.74±0.51** (*n*=66)		**6.26±0.33** (*n*=152)		**4.35±0.51** (*n*=66)	8.35×10^{-5}	5.54×10^{-3}	4.25
		Females	D1Mit403		**4.96±0.55** (*n*=37)		**3.34±0.40** (*n*=70)		**1.90±0.55** (*n*=37)	1.16×10^{-3}	5.89×10^{-2}	8.39
		Males	D1Mit403		**10.87±0.89** (*n*=29)		**9.34±0.53** (*n*=82)		**7.31±0.89** (*n*=29)	0.301	NS	–
Parasites in spleen (arbitrary units)	*Lmr5*	Both sexes	D10Mit12	**2.09**	5.34±0.14 (*n*=60)	**0.98**	4.58±0.09 (*n*=150)	**0.96**	4.56±0.12 (*n*=79)	8.09×10^{-5}	5.39×10^{-3}	4.54
		Females	D10Mit12	**1.60**	5.07±0.20 (*n*=31)	**0.60**	4.10±0.14 (*n*=69)	**0.52**	3.96±0.17 (*n*=46)	8.57×10^{-5}	5.76×10^{-3}	11.74
		Males	D10Mit12	**2.57**	5.55±0.20 (*n*=29)	**1.70**	5.14±0.12 (*n*=81)	**2.15**	5.37±0.18 (*n*=33)	0.173	NS	–
Splenomegaly (spleen-to-body weight ratio× 1,000)	*Lmr5*	Both sexes	D10Mit12		**21.43±0.61** (*n*=62)		**18.82±0.39** (*n*=151)		**17.49±0.53** (*n*=82)	8.63×10^{-6}	6.98×10^{-4}	11.60
			D10Mit46		**20.64±0.63** (*n*=61)		**18.76±0.40** (*n*=151)		**17.52±0.56** (*n*=76)	1.27×10^{-3}	6.21×10^{-2}	4.32
		Females	D10Mit12		**22.59±0.86** (*n*=33)		**19.06±0.59** (*n*=70)		**16.91±0.71** (*n*=48)	6.48×10^{-6}	5.57×10^{-4}	14.47
			D10Mit46		**22.56±0.96** (*n*=28)		**19.42±0.59** (*n*=73)		**17.27±0.72** (*n*=49)	1.06×10^{-4}	7.08×10^{-3}	11.50
		Males	D10Mit12		**19.42±0.84** (*n*=29)		**18.29±0.50** (*n*=81)		**17.71±0.77** (*n*=34)	0.318	NS	–
			D10Mit46		**18.78±0.78** (*n*=33)		**18.07±0.51** (*n*=78)		**18.34±0.87** (*n*=27)	0.750	NS	–

Mean and SE values were obtained by analysis of variance. Only linkages significant at whole genome level in the total group or in at least in one sex are given. The parasite loads were estimated by PCR–ELISA (see Materials and methods). In order to obtain normal distribution required for analysis of variance, value of parasites in spleens was transformed to the natural logarithm of (value×100). Splenomegaly (spleen-to-body weight ratio×1,000) and parasite load in draining lymph nodes exhibited normal distribution without transformation. The numbers in bold give the average non-transformed values. C and S indicate the presence of BALB/c and STS allele, respectively; *n* indicates number of mice.

Table 4 Loci controlling skin lesion development after infection with *L. major* in both sexes and separately in female and male F_2 hybrids between CcS-11 and BALB/c

Locus	Group	Marker	Genotype			*P* value	Corrected *P* value
			CC	CS	SS		
Lmr5	Both sexes	D10Mit12	**79.09±1.61** (*n*=62)	**71.46±1.03** (*n*=151)	**59.18±1.40** (*n*=82)	1.16×10^{-6}	5.27×10^{-5}
		D10Mit46	**79.84±1.64** (*n*=61)	**70.94±1.04** (*n*=151)	**58.90±1.47** (*n*=76)	1.05×10^{-6}	4.83×10^{-5}
	Females	D10Mit12	**79.06±2.29** (*n*=33)	**66.16±1.58** (*n*=70)	**52.01±1.91** (*n*=48)	6.34×10^{-6}	2.67×10^{-4}
		D10Mit46	**79.19±2.52** (*n*=28)	**66.60±1.56** (*n*=73)	**52.26±1.91** (*n*=49)	1.88×10^{-5}	7.28×10^{-4}
	Males	D10Mit12	**79.12±2.15** (*n*=29)	**76.03±1.29** (*n*=81)	**69.23±1.98** (*n*=34)	0.147	NS
		D10Mit46	**80.39±2.03** (*n*=33)	**75.00±1.32** (*n*=78)	**70.89±2.24** (*n*=27)	0.207	NS
Lmr21	Both sexes	D7Mit282	**109.53±3.63** (*n*=77)	**102.90±2.64** (*n*=140)	**86.35±3.98** (*n*=63)	4.3×10^{-3}	0.0972 (suggestive)
		D7Mit67	**113.18±3.58** (*n*=79)	**101.54±2.69** (*n*=132)	**85.39±3.53** (*n*=77)	4.25×10^{-4}	0.0116
	Females	D7Mit282	**103.20±4.92** (*n*=45)	**93.89±3.63** (*n*=80)	**81.06±6.57** (*n*=25)	0.1012	NS
		D7Mit67	**110.14±4.62** (*n*=46)	**91.70±3.62** (*n*=71)	**76.65±5.44** (*n*=32)	1.8×10^{-3}	0.0458
	Males	D7Mit282	**109.67±5.18** (*n*=32)	**112.75±3.79** (*n*=60)	**98.87±4.76** (*n*=38)	0.0895	NS
		D7Mit67	**113.89±5.39** (*n*=33)	**112.31±3.84** (*n*=61)	**99.95±4.45** (*n*=45)	0.172	NS

Mean and SE values were obtained by analysis of variance. Lesion size (mm^2) exhibited normal distribution without transformation. C and S indicate the presence of BALB/c and STS allele, respectively; *n* indicates number of mice. The values for *Lmr5* were calculated by analysis of covariance from all five time points (from 4 to 8 weeks of infection) as described in Materials and methods; the values for *Lmr21* by analysis of variance for week 8.

numbers in lymph nodes and in spleen are controlled independently: the locus *Lmr20* on chromosome 1 influences parasite load in draining lymph nodes, whereas parasite load in spleen is determined by the locus *Lmr5* on chromosome 10. *Lmr20* is not involved in regulation of skin lesion size, splenomegaly, or hepatomegaly, but it influences systemic immunological changes: it controls serum IgE levels in females, and it interacts with *Lmr23* on chromosome 16 in regulation of IFNγ level in serum.

The control of spread of parasites to spleen is exercised by another locus—*Lmr5*. In females, *Lmr5* controls parasite numbers in spleen and splenomegaly. This locus also influences pathology of another organ (the skin lesions). It has an impact on the systemic immune response by influencing serum IgE level, and in interactions with *Lmr21* on chromosome 7 and *Lmr22* on chromosome 12, also serum levels of IFNγ and IL-4, respectively. The BALB/c allele of *Lmr5* is associated with a larger parasite load, splenomegaly, and skin lesion size as well as higher IgE levels.

Table 5 Loci controlling IgE level in *L. major* infected F_2 hybrids between CcS-11 and BALB/c

Locus	Group	Marker	Genotype						*P* value	Corrected *P* value	Percent of explained variance
			CC		CS		SS				
Lmr20	Both sexes	D1Mit403	**21.63**	5.39±0.20 (*n*=69)	**16.86**	4.75±0.13 (*n*=157)	**12.69**	4.05±0.20 (*n*=69)	4.35×10^{-5}	3.07×10^{-3}	6.09
	Females	D1Mit403	**19.87**	5.17±0.25 (*n*=39)	**13.25**	4.15±0.18 (*n*=73)	**10.00**	3.49±0.24 (*n*=41)	2.36×10^{-5}	1.81×10^{-3}	11.76
	Males	D1Mit403	**18.54**	4.99±0.33 (*n*=30)	**17.44**	4.83±0.20 (*n*=84)	**13.86**	4.26±0.34 (*n*=28)	0.235	NS	–
Lmr5	Both sexes	D10Mit12	**18.03**	4.92±0.22 (*n*=62)	**16.19**	4.65±0.14 (*n*=150)	**11.82**	3.88±0.19 (*n*=81)	5.58×10^{-4}	3.02×10^{-2}	4.70
	Females	D10Mit12	**17.25**	4.81±0.28 (*n*=33)	**13.90**	4.27±0.19 (*n*=70)	**11.49**	3.81±0.24 (*n*=47)	2.98×10^{-2}	NS	–
	Males	D10Mit12	**18.38**	4.97±0.33 (*n*=29)	**18.78**	5.02±0.20 (*n*=80)	**11.92**	3.90±0.30 (*n*=34)	8.64×10^{-3}	NS	–

Mean and SE values were obtained by analysis of variance. In order to obtain normal distribution required for analysis of variance value of IgE in serum (μg/ml) was transformed by natural logarithm of value to the power of 1.5. The numbers in bold give the average non-transformed values. C and S indicate the presence of BALB/c and STS allele, respectively; *n* indicates number of mice.

Table 6 Interactions between loci (*Lmr20* and *Lmr23*) that control level of IFNγ in serum in *L. major* infected F_2 hybrids between CcS-11 and BALB/c

		Expl. var.=6.93%		$P=5.15\times10^{-4}$		Corr. $P=2.01\times10^{-2}$	
		D1Mit403 (*Lmr20*)					
D16Mit73 (*Lmr23*)			CC		CS		SS
	CC	**14.03**	1.63±0.11 (*n*=11)	**12.05**	1.58±0.06 (*n*=37)	**7.48**	1.42±0.10 (*n*=16)
	CS	**15.68**	1.66±0.06 (*n*=35)	**7.89**	1.44±0.05 (*n*=69)	**6.34**	1.36±0.09 (*n*=20)
	SS	**4.36**	1.21±0.11 (*n*=12)	**6.75**	1.38±0.07 (*n*=33)	**16.95**	1.68±0.10 (*n*=16)

Mean and SE values were obtained by analysis of variance. The linkage significant at whole genome level is given. In order to obtain normal distribution, we used natural logarithm of (IFNγ in serum [ng/ml] to the power of 0.5) and natural logarithm (IL-4 in serum [ng/ml] to the power of 1.1). The numbers in bold give the average non-transformed values. C and S indicate the presence of BALB/c and STS allele, respectively; *n* indicates number of mice.

The second locus that controls development of the skin lesions, *Lmr21* on chromosome 7, is not involved in control of parasite load in the lymph nodes nor in the spleen. Loci *Lmr22* (chromosome 12) and *Lmr23* (chromosome 16) control only systemic immune responses—serum IL-4 and IFNγ level, respectively. They do not affect parasite load, splenomegaly, or skin lesions.

It is important to understand that, as in any QTL study, failure to find a linkage between a phenotype and a marker does not rule out that such linkage may exist, although its phenotypic effect are likely smaller than in the detected linkages. So for a QTL, which affects several but not all parameters of a complex disease, this indicates that it has predominant effects on some parameters, although it might modify to a lesser extent other parameters as well.

Most loci we describe here affect several phenotypic traits. It is possible that some of these loci contain several linked genes, each affecting a different trait or a different subset of these traits. This is an important issue that needs to be resolved by recombinational analysis, as postulation of separate but linked loci merely on basis of a segregation study could be erroneous because of likely confounding random variations.

Control of parasite elimination differs between organs The loci *Lmr20* and *Lmr5* control differentially parasite numbers in lymph nodes and spleen, respectively, suggesting a predominantly organ-specific control of parasite load. This hypothesis is compatible with the mechanistic studies of others. The enzymes inducible nitric oxide synthase and phagocyte NADPH oxidase, which are required for the control of *L. major*, display organ- and stage-specific anti-*Leishmania* effects (Stenger et al. 1996; Blos et al. 2003). Inducible nitric oxide synthase has been shown to control resistance to parasites in skin and draining lymph nodes, but not in spleen of resistant strain C57BL/6 (Stenger et al. 1996). On the other hand, activity of phagocyte NADPH oxidase is essential for the clearance of *L. major* in the spleen, but it is dispensable for the resolution of the acute skin lesions and it exerted only a limited effect on the containment of the parasites in the draining lymph node (Blos et al. 2003). Similarly, the genes *Lmr20* and *Lmr5*

Table 7 Interactions between loci (*Lmr21* and *Lmr5*) that control level of IFNγ in serum in *L. major* infected F_2 hybrids between CcS-11 and BALB/c

		Expl. var.=7.18%		$P=4.96\times10^{-4}$		Corr. $P=1.95\times10^{-2}$	
		D7Mit67 (*Lmr21*)					
D10Mit12 (*Lmr5*)			CC		CS		SS
	CC	**4.86**	1.26±0.09 (*n*=18)	**13.66**	1.62±0.07 (*n*=25)	**21.93**	1.76±0.11 (*n*=11)
	CS	**11.81**	1.57±0.06 (*n*=34)	**8.86**	1.48±0.05 (*n*=57)	**11.71**	1.57±0.07 (*n*=30)
	SS	**11.59**	1.57±0.09 (*n*=16)	**6.71**	1.38±0.07 (*n*=30)	**4.91**	1.26±0.09 (*n*=17)

Mean and SE values were obtained by analysis of variance. The linkage significant at whole genome level is given. In order to obtain normal distribution, we used natural logarithm of (IFNγ in serum [ng/ml] to the power of 0.5) and natural logarithm (IL-4 in serum [ng/ml] to the power of 1.1). The numbers in bold give the average non-transformed values. C and S indicate the presence of BALB/c and STS allele, respectively; *n* indicates number of mice.

Table 8 Interactions between loci that control level of IL-4 in serum in *L. major* infected F_2 hybrids between CcS-11 and BALB/c

		Expl. var.=7.71%		$P=7.04\times10^{-4}$		Corr. $P=2.69\times10^{-2}$	
		D10Mit12 (*Lmr5*)					
D12Mit37 (*Lmr22*)			CC		CS		SS
	CC	**9.04**	2.38±0.38 (*n*=6)	**3.57**	1.30±0.19 (*n*=24)	**5.90**	1.88±0.23 (*n*=17)
	CS	**4.57**	1.58±0.20 (*n*=23)	**7.77**	2.20±0.13 (*n*=55)	**4.15**	1.48±0.21 (*n*=20)
	SS	**5.10**	1.71±0.23 (*n*=17)	**7.09**	2.09±0.18 (*n*=26)	**3.42**	1.26±0.24 (*n*=15)

Mean and SE values were obtained by analysis of variance. The linkage significant at whole genome level is given. In order to obtain normal distribution, we used natural logarithm of (IFNγ in serum [ng/ml] to the power of 0.5) and natural logarithm (IL-4 in serum [ng/ml] to the power of 1.1). The numbers in bold give the average non-transformed values. C and S indicate the presence of BALB/c and STS allele, respectively; *n* indicates number of mice.

might operate in different pathways of parasite killing or their function might be exerted by mediators produced by cells that influence these pathways.

Control of cytokine levels and its relationship to organ pathology and parasite numbers Several *Lmr* loci control IFNγ levels in combination with organ or skin pathology and parasite numbers. Different loci control different groups of phenotypes: *Lmr5*—organ pathology, parasite numbers in spleen, IFNγ level, and IL-4 levels; *Lmr20*—parasite numbers in lymph nodes and IFNγ level; *Lmr21*—organ pathology and IFNγ level; *Lmr23*—IFNγ only (For

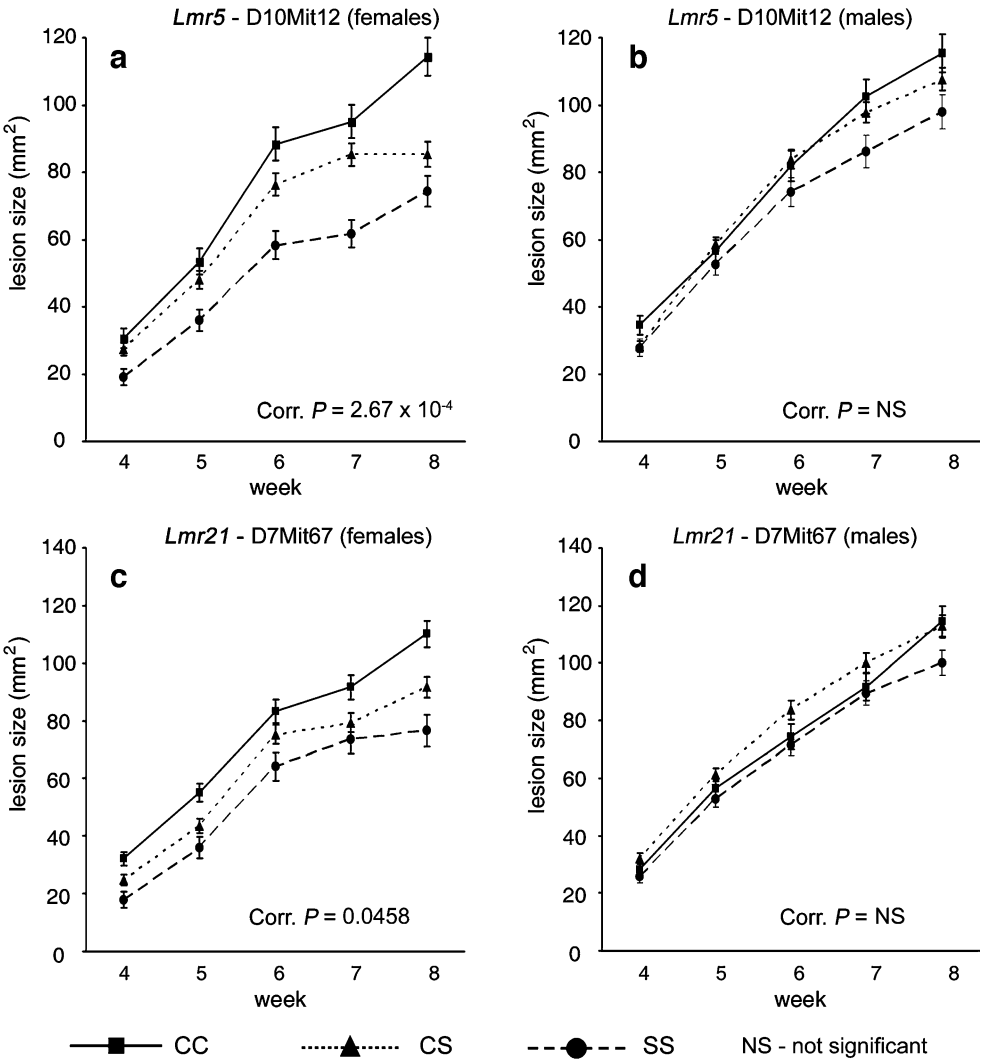

Fig. 2 Effects of genotype and sex on size of cutaneous lesion at *Lmr5* (**a**, **b**) and *Lmr21* (**c**, **d**) weeks4 to 8 after infection. The means and standard errors of means (*error bars*) for each of the three genotypes at different time points are given. The numbers of mice for D10Mit12 were 151 females and 144 males; for D7Mit67 149 females and 139 males. *C* and *S* indicate the presence of BALB/c and STS allele, respectively

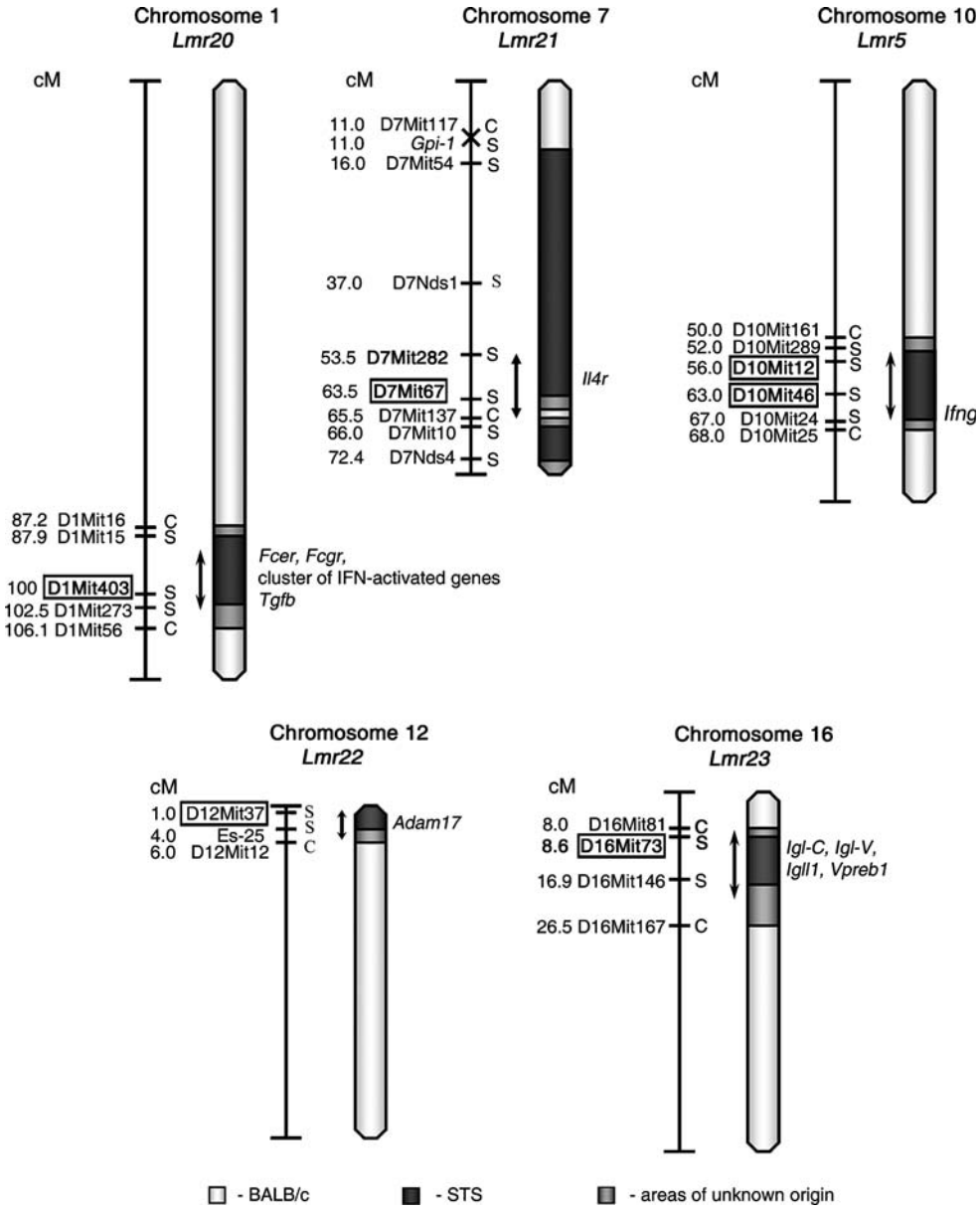

Fig. 3 Position of the loci that control response to *L. major* in strain CcS-11. The regions of STS and BALB/c origin are represented as *dark* and *white*, respectively; the boundary regions of undetermined origin are *shaded*. Only the markers defining the boundaries the STS-derived segment and the markers that were tested for linkage are shown. The markers that exhibit significant *P* values (corrected for genome-wide search) are shown in *boxes*. The *arrows* indicate the regions of significant linkage

interaction of these loci see Table 2). This suggests that pathology-controlling and/or parasite-controlling genes *Lmr5*, *Lmr21*, and *Lmr20* might operate via pathways that involve IFNγ. Some genes, such as *Lmr22* and *Lmr23*, are involved only in control of systemic immune response. These observations are in agreement with our previous detailed data on relationship of levels of IFNγ, IL-12, TNFα, IL-4 and IL-6, and organ pathology (Havelková et al. 2006) that indicated that there is no simple correlation between production of cytokines and development of disease. The influence of individual *Lmr* loci on several phenotypes can be due either to multiple effects of a single gene or these loci may comprise two or more closely linked genes with different effects.

Sex influence on parasite elimination, organ pathology, and systemic disease Our data show a sex influence on parasite numbers in secondary immune organs. Although strains BALB/c and CcS-11 did not exhibit any sex influence on lesion size, males of strain CcS-11 contained more parasites in spleens than females and males of both strains had much higher parasite load in lymph nodes. Previously, Mock and Nacy (1988) also reported higher parasite numbers in livers of males of BALB/cAnPt, DBA/2N, CDF1 and DBA/2J mice intravenously injected by *L. major*. These data collectively suggest that some genes controlling susceptibility to *L. major* might operate differently in the two sexes; however, we did not find instances of significant marker–sex interactions. *Lmr20* influences IgE level in serum only

Table 9 Potential candidate genes for *Lmr* loci controlling response to *L. major* in strain CcS-11

Chr.	Locus	Marker	Position in cM	Potential candidate gene	Position in cM
				Fcer1a, Fc receptor, IgE, high affinity I, alpha polypeptide (Lewis et al. 2004)	94.2
				Fcer1g, Fc receptor, IgE, high affinity I, gamma polypeptide (Lewis et al. 2004)	93.3
				Fcgr2b, Fc receptor, IgG, low affinity Iib (Padigel and Farrell 2005; Woelbing et al. 2006)	92.3
				Cluster of IFN activated genes (*Ifi201*, *Ifi202a*, *Ifi203*, *Ifi204*, *Ifi505*) (Blos et al. 2003)	95.2–95.3
1	*Lmr20*	D1Mit403	100	*Tgfb2* transforming growth factor, beta 2 (Padigel and Farrell 2005)	101.5
7	*Lmr21*	D7Mit67	63.5	*Il4r*, IL-4 receptor alpha chain (CD124) (Gessner et al. 1994)	62.0
10	*Lmr5*	D10Mit12	56.0		
		D10Mit46	63.0	*Ifng*, interferon gamma (Nacy et al. 1985)	67.0
12	*Lmr22*	D12Mit37	1.0	*Adam17* (a disintegrin and metalloproteinase domain 17) (Moss et al. 1997)	3.0
16	*Lmr23*	D16Mit73	8.6	Two clusters of genes that affect B-cell development (*Igl-C5*, *Igll1*, *Vpreb1*, *Vpreb2*; *Igl-C1*, *Igl-C2*, *Igl-C3*, *Igl-V1*, *Igl-V2*, *Igl1-r*) (Hoerauf et al. 1996; DeKrey et al. 2003)	9.8 13.0

in females. Genes controlling infections that appear to be sex dependent have been observed also with other pathogens. For example, *Rmp4* (resistance to mouse pox 4) controls susceptibility to ectromelia virus in female mice only (Brownstein and Gras 1995) and *Hrl* (herpes resistance locus) exhibits higher influence on susceptibility to Herpes simplex virus in male than in female mice (Lundberg et al. 2003). Sex-specific QTLs influence also susceptibility to Theiler's murine encephalomyelitis virus-induced demyelination: loci *Tmved7* and *8* affect male mice only, whereas locus *Tmved9* controls susceptibility only in females. Locus *Tmved6* operates both in females and males,

Table 10 Co-mapping with loci controlling other infections and other possibly related QTLs

Chr.	Locus	Marker	Position in cM	Co-localization with other possibly related QTLs	Position in cM
				Tir3c, trypanosomiasis infection response 3c (Iraqi et al. 2000)	93.0
				Ssta2, susceptibility to *Salmonella typhimurium* antigens 2 (Trezena et al. 2002)	95.8
1	*Lmr20*	D1Mit403	100	*Bbaa12*, *Borrelia burgdorferi*-associated arthritis 12 (Roper et al. 2001)	96.0
				berr1, Berghei resistance locus 1 (Bagot et al. 2002)	102.0
				Tria4, T-cell receptor induced activation 4 (Havelková et al. 1999)	46.0
				Heal6, wound healing/regeneration 6 (Mcbrearty et al. 1998)	52.4
				Tsiq1, T-cell secretion of IL-4 QTL1 (Choi et al. 2005)	59.0
7	*Lmr21*	D7Mit67	63.5	*Dice2*, determination of interleukin 4 commitment 2 (Bix et al. 1998)	63.5
12	*Lmr22*	D12Mit37	1.0	*Cinda2*, cytokine-induced activation (Krulová et al. 1997)	1.0
				Cd4ts5, CD4 T-cell subset 5 (Jackson et al. 2003)	6.0
16	*Lmr23*	D16Mit73	8.6	*Dice1*, determination of interleukin 4 commitment 1 (Bix et al. 1998; Baguet et al. 2004)	8.0

but it has an opposite effect on disease susceptibility in males and females (Butterfield et al. 2003).

Potential candidate genes The *Lmr* loci described here are mapped with a precision of 6–30 cM. The regions of the most probable linkage (estimated as the distance of markers that exhibit no significant linkage but are the closest to the markers with linkage) were between 4 and 19 cM long (Fig. 3), which is customary for the newly detected loci (Flint et al. 2005). These broad loci contain each several genes that have been shown previously to influence infection with *L. major* or that also influence responses to other pathogens as well as some immunological traits (for references, see Tables 9 and 10); however, the effects of many of *Lmr* loci might be caused by genes that are presently not suspected candidates. Presently, we are producing mice with recombinant haplotypes that carry each *Lmr* locus in a very short segment on chromosome. The testing of these strains will restrict the present number of the candidate genes to the most likely ones.

In summary, we show that *L. major* parasite load and disease manifestations are controlled by distinct sets of genes. Other genes also determine important immunological and pathological phenotypes of infected mice, but they do not appreciably affect parasite burden. By indicating which of the loci that control immune response and/or organ pathology are involved also in elimination or suppression of the parasite and which are not, these results represent an important step in a progress towards integration of genetic data and results from functional immunological studies.

An important corollary of these findings is that in humans there also likely exist polymorphic genes that control numbers of parasites and their organ distribution as well as other genes that affect some components of immune response and/or development or organ pathology but not parasite load. These polymorphisms will result in important individual differences in the "functional architecture" of the disease, its progression being in different individuals driven by different pathways. An apparently identical clinical phenotype in different individuals therefore can result from different functional pathways, and hence could differ in prognosis and response to therapy.

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III. The protective effect against *Leishmania* infection conferred by sand fly bites is limited to short-term exposure

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Leishmania are naturally transmitted by phlebotomine sand flies. Parasites get to the body of the host during an insect bite along with certain amount of sand fly saliva. In the endemic areas, people are regularly exposed to bites of uninfected sand flies; however sand fly numbers, and consequently the frequency of bites, vary during different periods of the year. It was hypothesized that people may gain more resistance against *Leishmania* being vaccinated against vector saliva. This concept was proven in various animal models, though such level of protection has not been reported from the field and there is lack of complex human studies. Despite of continuous exposure of the individuals to the insect bites, leishmaniasis still persists in the endemic areas. In our present study, four groups of BALB/c mice were exposed to *Phlebotomus duboscqi* bites following four different schemes, mimicking short- and long-term exposure to the saliva during different sand fly seasons. After such immunization, they were infected with *L. major* through inoculation into the ear dermis and analyzed for disease manifestations.

- ✓ Several parameters were tested: lesion size, parasite load in lymph nodes and infected ears, and specific anti-saliva IgG.

- ✓ Only mice, which were vaccinated with sand fly saliva shortly before the infection (group 2), developed higher resistance to *Leishmania*.

- ✓ Cumulative parasite load and lesion development were significantly lower in these mice (the group 2).

- ✓ Only the protected group (group 2) had significantly lower anti-*L. major* IgG levels compared with non-immunized mice, correlating with the lowest parasite load in this group.

- ✓ In mice that received long-term immunization (groups 15 and 15+0) or immunized two weeks before the infection (group 2+0), the protective effect was lost.

As one of the authors of this publication, I measured parasite load in organs of the infected mice using PCR-ELISA.

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Rapid Communication

The protective effect against *Leishmania* infection conferred by sand fly bites is limited to short-term exposure

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ABSTRACT

Under laboratory conditions, hosts exposed twice to sand fly saliva are protected against severe leishmaniasis. However, people in endemic areas are exposed to the vector over a long term and may experience sand fly-free periods. Therefore, we exposed mice long- or short-term to *Phlebotomus duboscqi* bites, followed by *Leishmania major* infection either immediately or after a sand fly-free period. We showed that protection against leishmaniasis is limited to short-term exposure to sand flies immediately before infection. Our results may explain the persistence of leishmaniasis in endemic areas and should be taken into account when designing anti-*Leishmania* vaccines based on sand fly saliva.

Leishmania spp. are intracellular protozoan parasites which infect mammalian phagocytic cells. The clinical manifestations of their multiplication and associated immunopathology depend on parasite species, genetic background and immune status of the host, ranging from cutaneous to fatal visceral disease. *Leishmania major* is a typical zoonotic species causing the cutaneous form of the disease. The transmission occurs predominantly between the vector and wild rodents (e.g. sand rats) with humans occasionally breaking into the zoonotic transmission cycle (Peters and Killick-Kendrick, 1987). The sole vectors of *Leishmania* parasites are female sand flies (Diptera: Phlebotominae), tiny bloodsucking insects widespread in the subtropics and tropics. During blood feeding, the parasites are deposited into host skin together with the fly saliva. Sand fly saliva contains antihaemostatic molecules and factors which modify the immune milieu of the skin. Not only do these components facilitate the acquisition of blood, but their presence in the infection site is also important for effective establishment of *Leishmania* parasites (Rohousova and Volf, 2006).

Individuals exposed to sand fly bites develop a specific immune response to salivary proteins (Rohousova and Volf, 2006). It was hypothesized that the enhancing effect of sand fly saliva on *Leishmania* infection could be abolished by this immune response in humans vaccinated against vector saliva, thereby preventing the establishment of infection. This concept has been demonstrated for *Leishmania* spp. causing both cutaneous and visceral leishmaniasis. Partial protection against *L. major* infection has been achieved in mice immunized by the bites of uninfected sand flies (Kamhawi et al., 2000), by salivary gland homogenate (Belkaid et al., 1998) or individual salivary components (Morris et al., 2001; Valenzuela et al., 2001; Oliveira et al., 2008). Protection has generally been associated with the production of IFN-γ and IL-12 upon challenge with *Leishmania* and saliva (Kamhawi et al., 2000; Gomes et al., 2008; Oliveira et al., 2008), suggesting that the existing anti-saliva delayed-type hypersensitivity (DTH) immune response creates an inhospitable environment for parasite survival.

While previous exposure of animals to sand fly feeding under laboratory conditions interferes with subsequent growth of transmitted *Leishmania* parasites, such protection has not been reported from the field. In endemic areas, the prevalence of *Leishmania* infection within the sand fly population is relatively low (Peters and Killick-Kendrick, 1987) and hosts are mostly exposed to the bites of uninfected sand flies. Despite this continuous exposure, leishmaniasis persists in endemic areas. Local inhabitants develop a specific antibody response to salivary antigens (Gomes et al., 2002; Rohousova et al., 2005; de Moura et al., 2007), which correlates with protection against visceral leishmaniasis (Gomes et al., 2002) but not against cutaneous leishmaniasis (Rohousova et al., 2005; de Moura et al., 2007). Clearly, the type of immune response elicited by laboratory immunization schemes and continuous exposure to uninfected sand flies occurring in the field are different.

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Thus, in our study we developed a mouse model of natural sand fly exposure to compare the outcome of infection in mice immunized using the exposure scheme previously reported (Belkaid et al., 1998; Kamhawi et al., 2000; Thiakaki et al., 2005) and in mice exposed "naturally", for a prolonged period.

Mice were bitten by *Phlebotomus duboscqi* and subsequently infected by *L. major*, the sole *Leishmania* sp. naturally transmitted by this sand fly sp. (Peters and Killick-Kendrick, 1987; Killick-Kendrick, 1990). The exposure schemes used in our study also address the seasonal dynamics of sand fly populations (Peters and Killick-Kendrick, 1987) and the persistence of the humoral immune response to sand fly saliva throughout the sand fly-free period.

All animals used in this study were maintained and handled strictly in accordance with institutional guidelines and Czech legislation (Act No. 246/1992 coll. on Protection of Animals against Cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals. The experiments were approved by the Committee on the Ethics of Animal Experiments of the Charles University in Prague (Permit Number: 24773/2008–10001) and were performed under the Certificate of Competency (Registration Number: CZU 934/05) in accordance with the Examination Order approved by Central Commission for Animal Welfare of the Czech Republic. Mice were anaesthetized by i.p. injection of 150 mg/kg ketamine and 15 mg/kg xylazine during the exposure phase and infection. All efforts were made to minimize the number and suffering of experimental animals within the study.

BALB/c mice were maintained in the animal facility of Charles University in Prague. Preliminary experiments were carried out to determine the optimal infection dose and exposure scheme. For the main study, 30 female mice (4 weeks old) were divided into five groups and exposed to *P. duboscqi* females (colony originating from Senegal). At each exposure, 30 female sand flies were allowed to feed on whole mouse body (for exposure schemes see Fig. 1A) with an average of 27 fed sand flies per mouse. The mice were subsequently infected intradermally in the right ear pinna with 10^4 *L. major* promastigotes (MHOM/IL/67/LRC-L137 JERICHO II) together with ¼ gland pair equivalent of *P. duboscqi* salivary gland homogenate in 5 μl saline. Lesions were measured using a digital caliper. Seven weeks after the infection, mice were sacrificed and sampled for blood, infected ears and draining lymph nodes. In total, mice were followed for 37 weeks.

Specific anti-*P. duboscqi* saliva IgGs were measured by immunoblot and ELISA as previously described (Rohousova et al., 2005). Immunoblots were performed on salivary gland homogenate separated by SDS–PAGE on 12.5% gel under non-reducing conditions. An equivalent of 50 gland pairs was loaded. After transfer, the membrane was cut into strips and incubated with mouse sera diluted 1:100 and goat anti-mouse IgG peroxidase-conjugate (heavy chain-specific, Sigma–Aldrich) diluted 1:750. For ELISA tests, wells were coated with *P. duboscqi* salivary gland homogenate (1/40 gland pair equivalent), sera were diluted 1:200 and goat anti-mouse IgG peroxidase-conjugate diluted 1:1000. The same ELISA protocol was used to measure specific anti-*L. major* IgG with two minor modifications: wells were coated with crude *L. major* promastigotes (10^6 cell equivalents per well) and sera were diluted 1:400.

Parasite burdens in the infected ear and draining lymph node were quantified by PCR-ELISA using a protocol described earlier (Kobets et al., 2010). The following primers were used: digoxigenin-labeled F 5′-ATT TTA CAC CAA CCC CCA GTT-3′ and biotin-labeled R 5′-GTG GGG GAG GGG CGT TCT-3′ (VBC-Genomics Biosciences Research, Austria). The cumulative parasite load was calculated as the sum of the parasite loads in both tested tissues.

Lesion size development was analyzed by general linear models (GLM) ANOVA and Scheffe's Multiple Comparison Procedure after data transformation ($\ln_{(x+1)}$). Other data (parasite load and antibody production) were subjected to non-parametric Wilcoxon tests. For correlation tests we used the non-parametric Spearman Rank Correlation Matrix. Statistical analyses were performed using NCSS 6.0.21 software.

Leishmania infection was monitored in BALB/c mice exposed to *P. duboscqi* bites following four different exposure schemes (Fig. 1A). Groups 2 and 15 represented short- and long-term exposures during the sand fly season with subsequent *Leishmania* transmission. Schemes used for groups 2 + 0 and 15 + 0 mimicked short- and long-term exposures followed by a sand fly-free period and *Leishmania* transmission occurring during the subsequent sand fly season.

In accordance with previous reports (Belkaid et al., 1998; Kamhawi et al., 2000; Thiakaki et al., 2005), *Leishmania* lesion size differed significantly between group 2 and control mice; in immunized mice the lesion size was smaller from week 3 p.i. onwards. Protection against *L. major* infection has previously been reported only for mice immunized by the saliva of *Phlebotomus papatasi* (Belkaid et al., 1998; Kamhawi et al., 2000). Here we report, to our knowledge for the first time, a similar protective effect in mice repeatedly bitten by *P. duboscqi*, the other proven vector of *L. major* (Peters and Killick-Kendrick, 1987; Killick-Kendrick, 1990). Although *L. major* established infection in immunized mice (group 2), the skin damage remained mild and the cumulative parasite load was significantly reduced by approximately threefold at week 7 p.i. (Fig. 1E). This finding corresponds with data published previously, where the number of parasites in *P. papatasi*-immunized mice was significantly reduced up to 8 weeks p.i. (Belkaid et al., 1998). The present study provides a model for further exploration of the vector-parasite-host immune interactions that influence establishment of *L. major* infection with mild or absent skin damage. Such a controlled amastigote population is expected to act as the sustained source of *L. major* being transmissible to another sand fly.

In some regions, sand fly populations vary greatly during the year, with sand flies occurring during several months of the year (Peters and Killick-Kendrick, 1987). To mimic this situation, mice of group 2 + 0 were exposed to *P. duboscqi* bites twice, similar to group 2, but with a subsequent delay of 15 weeks before the challenge with *L. major*. Compared with group 2, these mice showed significantly larger lesion size (Fig. 1B and D) and greater parasite load (Fig. 1E). All parameters of infection were equal to the control, non-immunized mice (Fig. 1B,D,E), strongly indicating that the protective effect was lost in those mice.

Long-term exposure to sand flies with possible desensitization to salivary antigens is another scenario that is likely to occur in endemic areas and is not reflected in studies published to date. To simulate this situation, the last two groups of mice were immunized 15 times, either directly before infection (group 15) or with a subsequent delay of 15 weeks before the challenge (group 15 + 0). Similar to group 2 + 0, the protective effect against the development of infection measured by lesion size was completely abrogated in both long-term exposure groups (Fig. 1B), with parasite load being comparable with that found in the control group (Fig. 1E). In the group 15 + 0, the parasite load in the infected ear was significantly higher than in the protected group (Fig. 1E). To date, desensitization to blood feeding insect saliva has only been described in mosquitoes (Peng and Simons, 1998). Here we show that a similar phenomenon may also occur in sand fly-infested areas, but the underlying mechanism needs to be elucidated. We can hypothesize that immunization with a large antigen load tends to skew the immune system towards a Th2 response which, in the case of groups 15 + 0 and 15, could be associated with the lack of

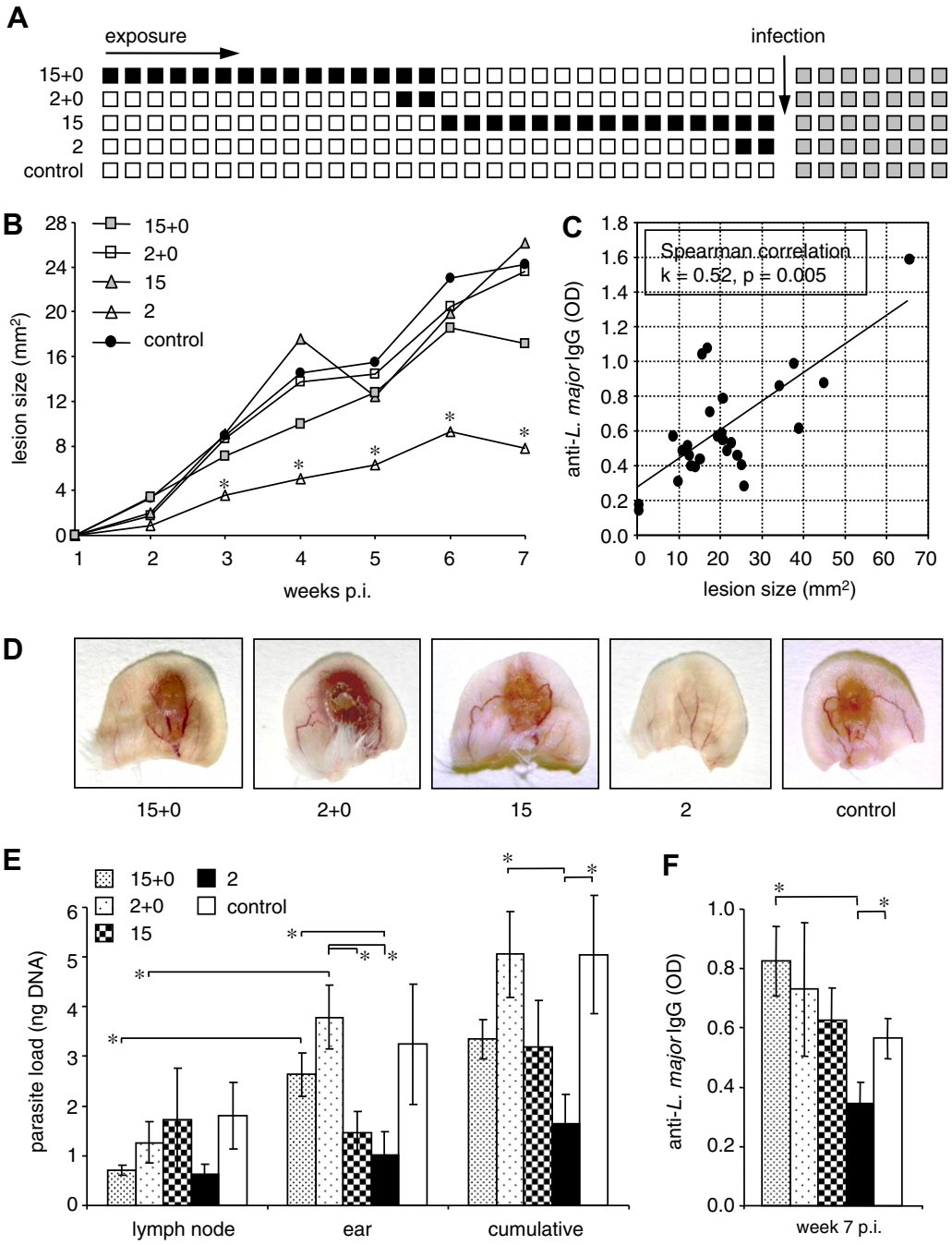

Fig. 1. Development of *Leishmania* infection. Main features of the mouse-based model set up to address the question of whether sand fly abundance and seasonality could result in different outcomes with respect to *Leishmania major* establishment and skin damage. (A) Different exposure schemes of BALB/c mice to sand flies. Each square represents 1 week when mice were exposed (■) or unexposed (□) to *Phlebotomus duboscqi* bites; mice were followed for 7 weeks p.i. with 10^4 *L. major* promastigotes plus half of a *P. duboscqi* salivary gland (■). (B) *Leishmania* lesion size was monitored weekly. Asterisks (∗) indicate the significant difference of group 2 from all other groups ($P < 0.05$). (C) Graph showing positive correlation between *Leishmania* lesion size and anti-*L. major* IgG antibodies measured at week 7 p.i. (D) Representative photographs of mouse ears from each group showing *Leishmania* lesion in infected ears at week 7 p.i. (E) At week 7 p.i., parasite load was measured in the infected ear and draining lymph node (ng of *L. major* DNA per 50 ng of total DNA). Cumulative parasite load is the sum of parasite load in both tissues. Error bars represent mean ± S.E., $^*P < 0.05$. (F) Anti-*L. major* IgG antibodies measured at week 7 p.i. Error bars represent mean ± S.E., $^*P < 0.05$.

protection. Additionally, the severity of skin damage could be due to the deposition of immune complexes in the skin, leading to an exacerbated immune response from the early stage of lesion development. These immune complexes are more likely to form between anti-saliva antibodies and salivary proteins since no binding was observed between anti-*P. duboscqi* saliva antibodies and *L. major* promastigotes (Rohousova et al., unpublished data).

All groups of mice showed elevated anti-*Leishmania* IgG at week 7 p.i. (Fig. 1F) with a positive correlation found between the levels of anti-*L. major* IgG and the lesion size (Fig. 1C). Accordingly, only the protected group (group 2) had significantly lower anti-*L. major* IgG levels compared with non-immunized mice (Fig. 1F), correlating well with the lowest parasite load in this group.

In areas where cutaneous leishmaniasis is endemic, the presence of active *Leishmania* lesions in patients is associated with elevated

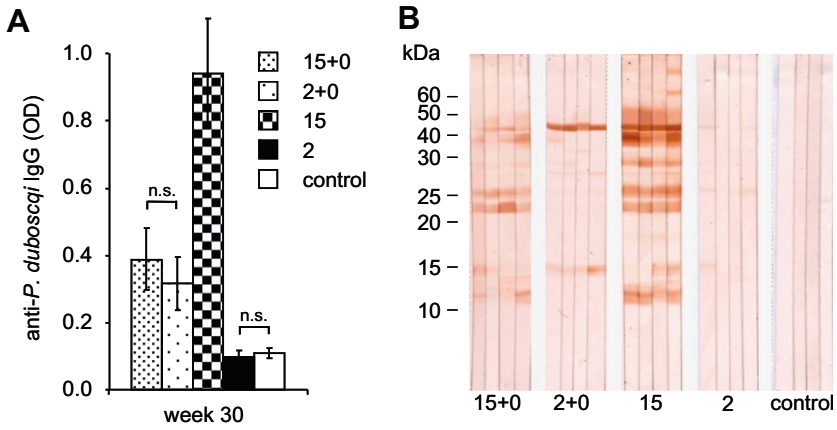

Fig. 2. Antibody response to *Phlebotomus duboscqi* salivary antigens. BALB/c mice were exposed to *P. duboscqi* bites following the scheme shown in Fig. 1A. Anti-*P. duboscqi* saliva IgG antibodies were measured at the end of immunization (week 30) in non-infected mice using ELISA (A) and immunoblot (B). Error bars represent mean ± S.E., n.s. means not significant ($P > 0.05$); the difference is significant ($P < 0.05$) between all other group combinations.

levels of anti-vector saliva IgG (Rohousova et al., 2005; de Moura et al., 2007), suggesting that these antibodies could be used as a risk marker for *Leishmania* transmission. Accordingly, we measured anti-*P. duboscqi* IgG levels and correlated those with the status of *Leishmania* infection. At the end of immunization (week 30, before infection), only groups 15 + 0, 2 + 0 and 15 had elevated levels of anti-*P. duboscqi* saliva IgG, with group 15 having the highest level (Fig. 2A) and strongly recognizing at least seven out of 12 protein bands within the broad range of 12–60 kDa (Fig. 2B). The immunoblot revealed that in group 15 + 0, the level of specific antibodies decreased similarly for all antigens. Mice in group 2 + 0 recognized only one or two antigens, with the protein band of approximately 42–45 kDa being the strongest antigen. Mice from groups 2 and control showed weak or no visible reaction with *P. duboscqi* salivary proteins (Fig. 2B). Within all tested groups, a positive correlation was found between anti-*P. duboscqi* IgG and *Leishmania* lesion parasite load ($k = 0.38$, $P = 0.04$), supporting the above mentioned hypothesis that anti-*P. duboscqi* IgG correlates with skin damage. On the other hand, no correlation was found between anti-*P. duboscqi* IgG and parasite load in the draining lymph node, indicating that pre-exposure to *P. duboscqi* bites could not alter parasite dissemination. Moreover, groups with a delay between pre-exposure and infection (groups 15 + 0 and 2 + 0) had significantly higher parasite loads in the ears than in the draining lymph nodes (Fig. 1E), indicating that in bitten hosts *L. major* preferentially multiplies in the skin tissue. The hypothesis that different immune regulatory mechanisms operate in those tissues is also supported with our previous study showing that parasite numbers in lymph nodes and development of skin lesions are under distinct genetic control (Kurey et al., 2009).

Anti-*P. duboscqi* saliva antibodies were undetectable or absent throughout the study in two groups – group 2 and unexposed control mice (Fig. 2). Taking into account the outcome of infection in these groups, we assume that one of the following applies to mice negative for anti-saliva antibodies: (i) the host is sand fly saliva-naive; therefore saliva in the infective inoculum would exacerbate the development of infection and accelerate skin damage, as in the control group, or (ii) the host has been recently immunized with a low dose of antigen, thus the antigen dose and/or the time-frame did not allow the production of detectable levels of specific anti-saliva antibodies. At the same time, the immune response resulting from this immunization scheme protects the host against leishmaniasis and although *L. major* establishes an infection, the skin damage remains mild (as in group 2). This assumption is in agreement with field results from an endemic area of cutaneous leishmaniasis caused by *Leishmania braziliensis*: low levels of anti-vector saliva IgG were found both in individuals without previous contact with *Leishmania* and in individuals positive for anti-*Leishmania* DTH, but apparently protected from lesion development (de Moura et al., 2007), possibly by short-term or low exposure to sand flies. A different situation has been reported from an endemic area of visceral leishmaniasis where anti-vector saliva antibodies in exposed individuals positively correlated with anti-parasite cell-mediated protective immunity (Gomes et al., 2002).

The concept of using sand fly salivary proteins in anti-*Leishmania* vaccines is based on reports of Belkaid et al. (1998) and Kamhawi et al. (2000), and has been reinforced by several studies testing particular salivary proteins (Morris et al., 2001; Valenzuela et al., 2001; Vinhas et al., 2007; Gomes et al., 2008; Collin et al., 2009). This concept is not rejected by our study, since proteins administered as a vaccine may prime a different immune response from those naturally deposited by sand flies (Plotkin, 2005). Moreover, the targeted host species, humans and dogs, are outbred and therefore more diverse in terms of resistance and susceptibility to leishmaniasis than one strain of inbred laboratory mice.

Our study on BALB/c mice attempts to test the limitations of the sand fly saliva-induced protective effect on *Leishmania* infection and several questions to be addressed were raised. (i) What is the kinetics of anti-saliva antibody and cellular immune responses? (ii) What is the mechanism underlying the loss of protective effect conferred by exposure to salivary antigens? Could antibodies block the protective effect as suggested by in vitro studies (Belkaid et al., 1998; Cavalcante et al., 2003)? (iii) Would saliva-induced protection be lost upon long-term exposure to sand flies even in other host species, e.g. mouse strains resistant to *Leshmania* infection or dogs?

In conclusion we described here, to our knowledge for the first time, limitations of the sand fly saliva-induced protective effect on the development of *Leishmania* infection. In previous studies, hosts protected by immunization with sand fly saliva (either by bite or by injection) were immunized twice at 1- or 2-week intervals and infected immediately thereafter (Belkaid et al., 1998; Kamhawi et al., 2000). Our results might help to explain the persistence of *Leishmania* infection in endemic areas and should be taken into account when designing and testing vaccines based on vector salivary proteins.

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IV. Genetic model for analysis of susceptibility to the parasite *Leishmania tropica* – parasites cause pathological changes in skin and, in some strains, can invade spleens

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In preparation...

Similarly as *L. major, L. tropica* causes cutaneous leishmaniasis in humans, but was also reported to visceralize and cause systemic illness. Relationship between the host genotype and disease manifestations has been poorly studied because of lack of the suitable animal models. On the background of our previous studies, we chose several RC mouse strains (CcS-3, -5, -11, -12, -16, -18 and 20), which exhibited various susceptibility to *L. major*, in order to establish a genetic model for susceptibility to *L. tropica.* The infected mice were tested for several parameters: skin lesion size, splenomegaly (enlargement of the spleen), hepatomegaly (enlargement of the liver), parasite load in inguinal lymph nodes and spleen, levels of IFNγ, IL-4, IL-12, IgE, GM-CSF, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) and CCL7 (MCP-3) in blood serum. Parasite load was measured using modified limiting dilution assay.

- ✓ In comparison to *L. major* experiments, the infection with *L. tropica* developed slowly.

- ✓ Draining lymph nodes of all tested stains contained viable parasites.
- ✓ *L. tropica* parasites visceralized and were found in spleens of strains BALB/c, CcS-3, CcS-18, and CcS-20. Dissemination of *Leishmania* to the spleen was confirmed by microscopy.
- ✓ Parasite load was much lower than after infection with *L. major.*
- ✓ Similarly as in *L. major* experiments, STS and CcS-5 strains showed the highest resistance to *L. tropica* induced disease.
- ✓ Nodules on skin at the site of infection appeared late, at the 11-th week of infection, only some of the tested CcS/Dem strains developed skin lesions (CcS-16, BALB/c, CcS-11, CcS-20).
- ✓ After infection the strains BALB/c, CcS-11, CcS-18, CcS-16 and CcS-20 have increased levels of chemokines CCL2, CCL3 and CCL5 in serum.
- ✓ We have observed strong sex influence that is different from that observed after infection with *L. major*. In *L. major* experiments, males exhibited either higher or similar pathology as females. In contrast, *L. tropica* infected females were more susceptible than males.
- ✓ The largest skin lesions were observed in CcS-16 females.
- ✓ Females of CcS-18 and CcS-12 developed only small skin pathology.
- ✓ Only males of BALB/c, CcS-16 and CcS-20 developed small lesions, whereas males of other strains had no skin pathology.
- ✓ CcS-16 females but not males exhibited a unique pattern of this systemic reaction, characterized by the additional early peak of chemokine levels.

- ✓ Despite of visceralization of parasites in several strains, we observed no splenomegaly or hepatomegaly.

- ✓ We were first who successfully established a reliable mouse model for genetic studies of *Leishmania tropica* infection.

As the first author, I contributed to the isolation of organs from the infected mice, improvement and optimization of the limiting dilution assay for quantification of parasites, measurement of parasite load in spleen and lymph nodes, performed examination of H&E stained histological sections and immunohistochemical staining, contributed to statistical analysis and interpretation of the results, and participated in writing of the manuscript.

Genetic Model for Analysis of Susceptibility to the Parasite *Leishmania tropica* – Parasites Cause Pathological Changes in Skin and, in Some Strains, Can Invade Spleens

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Running head: Genetic model for study of susceptibility to *Leishmania tropica*

Keywords: *Leishmania tropica*; immune response; pathology; parasite numbers in visceral organs; CCL5/RANTES

Abstract

Background: Leishmaniasis is a group of diseases caused by intracellular protozoan parasites of several *Leishmania* species. The importance of involvement of *Leishmania tropica* in human leishmaniasis has been recognized only recently. Similarly as *L. major, L. tropica* causes cutaneous leishmaniasis in humans, but was also reported to visceralize and cause systemic illness. Relationship between the host genotype and disease manifestations has been poorly studied because of lack of the suitable animal models.

Methods: In order to establish a genetic model for susceptibility to *L. tropica*, we used recombinant congenic (RC) strains of the BALB/c-c-STS/A (CcS/Dem) series (CcS-3, -5, -11, -12, -16, -18 and 20). These strains exhibit very large differences in susceptibility to *L. major*. Each RC series contains a different random subset of about 12.5% of genes from the parental donor strain STS/A and the remaining approximately 87.5% genes of the background strain BALB/c. Mice were infected with *L. tropica* and development of skin lesions, cytokine and chemokine levels in serum, and parasite numbers in organs were established.

Results: Genotype effects are different in *L. tropica* and *L. major* infection. After infection with *L. tropica* females of strains CcS-16, BALB/c, CcS-11 and CcS-20 are susceptible to development of skin lesions, larger lesions being observed in CcS-16. Females of CcS-18 and CcS-12 developed only small skin pathology and strains CcS-3, CcS-5 and STS were resistant to skin lesions development. Only males of BALB/c, CcS-16 and CcS-20 developed small skin lesions. This pattern is clearly different from those observed after infection with *L. major*, where both females and males of the strains BALB/c, CcS-12, CcS-16, and CcS-18 develop large lesions, CcS-3, CcS-11 and CcS-20 intermediate lesions and CcS-5 and STS are resistant to infection. We have observed strong sex influence that is different from that observed after infection with *L. major*. In *L. major* experiments, males exhibited either higher or similar pathology as females. In contrast, *L. tropica* infected

females were more susceptible than males. After infection the strains BALB/c, CcS-11, CcS-18, CcS-16 and CcS-20 have increased levels of chemokines CCL2, CCL3 and CCL5 in serum. Unexpectedly and in contrast with the other strains tested, the CcS-16 females but not males exhibited a unique pattern of this systemic reaction, characterized by an additional early peak of chemokine levels. Draining lymph nodes of all tested stains contained viable parasites, but the numbers were much lower than after infection with *L. major*. *L. tropica* parasites visceralized and were found in spleens of strains BALB/c, CcS-3, CcS-18, and CcS-20.

Conclusion: Comparison of *L. tropica* and *L. major* infections indicates existence of the species-specific patterns of response to infection, with different sex effects and different host susceptibility genes. The CcS strains animal model is a powerful tool to characterize the mechanisms of species specific as well as individual specific host-parasite interactions.

Author Summary

Several hundred million people are exposed to the risk of leishmaniasis, a disease caused by intracellular protozoan parasites of several *Leishmania* species and transmitted by phlebotomine sand flies. In humans, *L. tropica* causes cutaneous form of leishmaniasis with painful and long-persisting lesions in the site of the insect bite, but the parasites can also penetrate to the inner organs. Numerous studies of different infectious diseases demonstrated relationship between the host genes and development of the disease. For many reasons, search for susceptibility genes in human population could be a difficult task. In such cases, animal models may help to discover and prove the important role of different genes in interactions between the parasite and the host. Unfortunately, literature contains only a few publications about attempts to use animals for *L. tropica* studies. Here, we report a suitable animal model for genetic studies of *L. tropica* infection and show how the host genotype influences different disease symptoms: skin lesion development, parasite dissemination to the lymph nodes and spleen, and increased serum levels of chemokines CCL2, CCL3 and CCL5 in serum that characterizes development of inflammation in susceptible strains.

Introduction

Several hundred million people in 88 countries are currently exposed to the danger of leishmaniasis, a disease that is caused by intracellular protozoan parasites of the genus *Leishmania* and transmitted to vertebrates by phlebotomine sand flies. *Leishmania* parasites infect professional phagocytes (neutrophils, monocytes and macrophages), as well as dendritic cells and fibroblasts (Rittig and Bogdan 2000). The main host cell is the macrophage, where parasites multiply, eventually rupturing the cell and spreading to uninfected cells (Reiner and Locksley 1995). As macrophages are present in all tissues of the mammals, *Leishmania* parasites have a great potential for damaging bodily functions. In the dermis, they cause the cutaneous form of the disease (which can be localized or diffuse); in the mucosa, they result in mucocutaneous leishmaniasis; and the metastatic spread of infection to the spleen and liver leads to visceral leishmaniasis. One of the major factors determining the type of pathology is the species of *Leishmania* (McMahon-Pratt and Alexander 2004). However, the transmitting vector, as well as genotype and nutritional status of the host, environmental and social factors have also a large impact on the outcome of the disease (McMahon-Pratt and Alexander 2004, Farrell 2002). That is why even patients, infected by the same species of *Leishmania*, develop different symptoms (McMahon-Pratt and Alexander 2004) and may differ in their response to therapy (Berman et al. 1997). The basis of this variation is not well understood (Herwaldt 1999) but part of this variation is likely genetic. Numerous genes that might be involved were reported (Lipoldová and Demant 2006).

The importance of involvement of *Leishmania tropica* in human leishmaniasis has been recognized only recently. The foci of this parasite have been found on the Greek island Zakyntos, in Turkey, Syria, Jordan, Israel, Morocco, Tunisia, Saudi Arabia, Yemen, Iran, Iraq, Afghanistan, Turkmenistan, India, Pakistan, Kenya, Ethiopia and Namibia (Jacobson 2003). While *L. major* is a zoonosis with mainly rodent (*Gerbillidae*) reservoir hosts, *L. tropica* can circulate among humans without the involvement of animal reservoirs. Similarly

to *L. major, L. tropica* causes cutaneous leishmaniasis in humans. Moreover, *L. tropica* was also reported to visceralize and cause unexplained systemic illness in veterans returning from endemic areas in Middle East (Magill et al. 1993), the classical visceral leishmaniasis (kala-azar) in India (Sacks et al. 1995), and disseminated cutaneous leishmaniasis accompanied with visceral leishmaniasis in Iran (Alborzi et al. 2008). Therefore, a suitable animal model for study of this parasite would contribute to understanding of the clinical course of infection.

Golden hamsters (*Mesocritetus auratus*) have been considered to be the best model host of the *L. tropica,* but this host is not inbred. However, several strains of *L. tropica* have been described to cause cutaneous disease in inbred BALB/c mice (Lira et al. 1998, Girginkardesler et al. 2001), thus providing a better defined host. In comparison with widely studied immune response to *L. major* infection (Sacks and Noben-Traut 2002) and its genetic control (Lipoldová and Demant 2006), little is known about *L. tropica* in mouse (Lira et al. 1998, Girginkardesler et al. 2001, Anderson et al. 2008). Here we aimed to establish a genetic model to study susceptibility to *L. tropica*. We analyzed response to *L. tropica* in CcS/Dem (CcS) recombinant congenic (RC) strains (Demant and Hart 1986) derived from the background strain BALB/c and the donor strain STS. Each CcS strain contains a different unique random set of about 12.5% genes from the donor strain STS and 87.5% genes from the background strain BALB/c. These strains have been already successfully used for analysis of infection with *Leishmania major* (Demant et al. 1996, Lipoldová et al. 2000, Vladimirov et al. 2003, Havelková et al. 2006). This model enabled us to analyze organ pathology and systemic disease after infection with *L. tropica.*

Materials and Methods

Mice

Males and females of strains BALB/c, STS and selected recombinant congenic (RC) strains (Demant and Hart 1986, Stassen et al. 1996) were tested. When used for these experiments, RC strains were in more than 38 generation of inbreeding and therefore highly homozygous. During the experiment male and female mice were placed into separate rooms and males were caged individually.

In the experiment with *Leishmania tropica*, mice of the strains BALB/c (18 females, 12 males), STS (12 females, 13 males), CcS-3 (12 females, 13 males), CcS-5 (17 females, 12 males), CcS-11 (17 females, 19 males), CcS-12 (12 females, 6 males), CcS-16 (17 females, 12 males), CcS-18 (8 females, 12 males), and CcS-20 (12 females, 12 males) were infected with *L. tropica*. Mice were tested in 3 successive experimental groups and were killed 21, 32 and 43 weeks after infection. Age of the mice at the time of infection was 9 to 26 weeks (mean 16 weeks, median 16 weeks).

In the experiment with *Leishmania major,* mice of the strains BALB/c (25 females, 26 males), STS (8 females, 9 males), CcS-3 (10 females, 10 males), CcS-5 (23 females, 29 males), CcS-11 (18 females, 20 males), CcS-12 (19 females, 16 males), Ccs-16 (11 females, 13 males), CcS-18 (9 females, 4 males), and CcS-20 (13 females, 15 males) were infected. Mice were tested in 8 successive experimental groups and were killed 8 weeks after infection. Age of the mice at the time of infection was 8 to 47 weeks (mean 16 weeks, median 15 weeks).

Parasite

Leishmania tropica from Urfa, Turkey (MHOM/1999/TR/SU23) was used for infecting mice. 10^7 stationary phase promastigotes from subculture 2 have been inoculated in

50 μl of sterile saline s.c. into the tail base, with promastigote secretory gel (PSG) collected from *L. tropica*-infected *Phlebotomus sergenti* females (laboratory colony originating from Urfa focus). PSG was collected as described (Rogers et al. 2004). The amount corresponding to one sand fly female was used per mouse.

Leishmania major LV 561 (MHOM/IL/67/LRC-L137 JERICHO II) was maintained in rump lesions of BALB/c females. Amastigotes were transformed to promastigotes using SNB-9 (Diamond and Herman 1954); 10^7 promastigotes from 6 days old subculture 2 were inoculated in 50 μl of sterile saline s.c. into mouse rump.

Disease phenotype

Size of the primary skin lesions was measured weekly using a Vernier caliper gauge. The mice infected with *L. tropica* were killed 21, 32 or 43 weeks after inoculation. Mice infected with *L. major* were killed 8 weeks after infection. The blood, spleen, liver and inguinal lymph nodes were collected for the further analysis.

Quantification of parasite load in spleens and inguinal lymph nodes

The current semi quantitative technique is based on the limited dilution assay (Titus et al. 1985). We modified the procedure by using only a single pre-selected cell concentration, and parasite count was measured with a Coulter Counter (Coulter Electronics Inc., USA). In comparison with the original limited dilution technique, this modified culture method is less laborious and allows rapid estimation of parasite number.Preparation of cells must be carried out under sterile conditions. During preparation, all samples, which were not immediately worked with, were kept on ice.

Inguinal lymph nodes were disrupted in a glass homogenizer in complete RPMI (containing 5 % of inactivated fetal calf serum (Sigma-Aldrich, USA), 25 mM Hepes (Sigma-Aldrich, USA), 0.0005 % β-mercaptoethanol (Serva, Germany), 63.7 μg/ml penicillin

(Sigma-Aldrich, USA), and 100 μg/ml streptomycin (Sigma-Aldrich, USA). The homogenate was passed through the nylon filter. The homogenizer was washed 3 times with 3 ml of sterile PBS after processing each lymph node. The samples were then centrifuged 8 min at 300×g, 4 ^{o}C (centrifuge Eppendorf 5810 R, Eppendorf, Germany). Supernatant was removed and the cells were resuspended in 0.5 ml of complete Schneider's medium supplemented with 20 % inactivated fetal calf serum (Sigma-Aldrich, USA), 2 % sterile fresh human urine, 50 μg/ml gentamicine (Sigma-Aldrich, USA), 63.7 μg/ml penicillin (Sigma-Aldrich, USA), and 100 μg/ml streptomycin (Sigma-Aldrich, USA). To count cells with a Coulter Counter CBC5 (Coulter Electronics Inc., USA), 50 μl of the cell suspension was diluted in 20 ml of PBS. Ekoglobin (Hemax spol. s.r.o., Czech Republic) was added to the diluent prior to counting to lyse red blood cells.

For parasite counting, 0.5 ml of the cell suspension (1×10^5 cells per ml for lymph nodes and 2×10^5 cells per ml for spleens) was cultivated in complete Schneider's medium in 48-well tissue culture plates (Costar, Corning Inc., USA) at 27 ^{o}C (Biological thermostat BT 120 M, Labsystem, Finland) for 3 days. Each sample was prepared in triplicate. After incubation, 100 μl of a mixed sample from each well, containing *Leishmania* parasites released from lymph node or spleen cells, were diluted in 20 ml of PBS and the parasite number was counted with the Coulter Counter.

Cytokine and IgE levels

Levels of GM-CSF (granulocyte-macrophage colony-stimulating factor), CCL2 (chemokine ligand 2)/MCP-1 (monocyte chemotactic protein-1), CCL3/MIP-1α (macrophage inflammatory protein-1α), CCL4/MIP-1β (macrophage inflammatory protein-1β), CCL5/RANTES (regulated upon activation, normal T-cell expressed, and secreted) and CCL7/MCP-3 (monocyte chemotactic protein-3), in serum were determined using Mouse

chemokine 6-plex kit (Bender MedSystems, Austria). The kit contains two sets of beads of different size internally dyed with different intensities of fluorescent dye. The set of small beads is used for GM-CSF, CCL5/RANTES and CCL4/MIP-1β and set of large beads for CCL3/MIP-1α, CCL2/MCP-1 and CCL7/MCP-3. The beads are coated with antibodies specifically reacting with each of the analytes (chemokines) to be detected in the multiplex system. A biotin secondary antibody mixture binds to the analytes captured by the first antibody. Streptavidin – Phycoerythrin binds to the biotin conjugate and emits fluorescent signal. Test procedure was performed in the 96 well filter plates (Millipore, USA) according to the protocol of Bender MedSystem. Beads were analyzed on flow cytometer LSR II (BD Biosciences, USA). The limit of detection of each analyte was determined to be for GM-CSF 12.2 pg/ml, CCL2/MCP-1 42 pg/ml, CCL7/MCP-3 1.4 pg/ml, CCL3/MIP-1α 1.8 pg/ml, CCL4/MIP-1β 14.9 pg/ml, CCL5/RANTES 6.1 pg/ml respectively.

IFNγ, IL-4, IL-12 and IgE levels in serum were determined using the primary and secondary monoclonal antibodies (IFNγ: R4-6A2, XMG1.2; IL-4: 11B11, BVD6-24G2; IL-12p40/p70: C15.6, C17.8; IgE: R35-72**,** R35118) and standards from BD Biosciences, USA (recombinant mIFNγ, mIL-4, mIL-12 and purified mIgE: C38-2). The ELISA was performed as recommended by BD Biosciences. The ELISA measurement of IFNγ, IL-4, IL-12, and IgE levels was performed by the ELISA Reader Tecan and the curve fitter program KIM-E (Schoeller Pharma, Czech Republic) using least squares-based linear regression analysis. The detection limit of the ELISA was determined to be 30 pg/ml for IFNγ, 8 ng/ml for IgE, 16 pg/ml for IL-4 and 15 pg/ml for IL-2.

Staining of tissue sections

Inguinal lymph nodes and spleens were fixed in 4% formaldehyde and embedded in paraffin. Immunohistochemical staining of parasites was performed in 5 μm lymph node

sections. Slides were deparaffinized with xylene (2 times for 5 min) and rehydrated with 96 % ethanol (3 times for 3 min), 80 % ethanol (3 min), 70 % ethanol (3 min) and PBS (phosphate buffer saline, 3 min). Endogenous peroxidase was quenched with 3 % H_2O_2 in methanol for 10 min. Sections were washed in PBS (10 min) and parasites were stained using anti-*Leishmania* lipophosphoglycan monoclonal mouse IgM (Code Nr. CLP003A, Cedarlane, Canada) diluted 1:100 in PBS with 1 % BSA (bovine serum albumine, Sigma-Aldrich, USA) and applied for 1 h at 37 °C, followed by TRITC-conjugated goat anti-mouse IgM (Code Nr. 115-025-020, Jackson Immunoresearch, USA), also diluted 1:100 in PBS with 1 % BSA and applied for 1 h at 37 °C. Cell nuclei were stained with DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) 10 ng/µl (Sigma-Aldrich, USA). For histological analysis, 5 µm spleen and lymph node sections were stained by routine hematoxylin and eosin method (H&E).

Statistical analysis

The differences between CcS/Dem strains in parasite numbers in lymph nodes were evaluated by the analysis of variance (ANOVA) and Newman-Keuls multiple comparison using the program Statistica for Windows 8.0 (StatSoft, Inc., USA). Strain and age were fixed factors and individual experiments were considered as a random parameter. The differences in parameters between strains were evaluated using the Newman-Keuls multiple comparison test at 95% significance. Difference between sexes in parasite numbers in lymph nodes was calculated by Mann Whitney U test. Difference in skin lesion development and skin pathology between BALB/c and STS, and BALB/c and each of CcS/Dem strains was compared by Mann Whitney U test. Relationships between organ pathology and chemokine and IgE levels were evaluated by nonparametric Spearman correlation.

In experiments with *L. major*, differences in skin pathology between BALB/c and CcS/Dem strains were calculated with ANOVA, and differences between sexes were estimated with Mann-Witney U-test using Statistica.

Results

Optimization of infection protocol

To optimize the infection protocol, we determined the influence of inoculation site on host susceptibility and disease progress in BALB/c mice. 10^7 promastigotes from 6 days old subculture 2 have been inoculated in 10-50 µl of sterile saline s.c. into the tail base, hind foot, or ear. Mice inoculated into the tail base exhibited the most extensive pathology (data not shown) and this site of inoculation was therefore used in subsequent studies. We have also tested the influence of *L. tropica* co-inoculation with sand fly vector saliva or promastigote surface gel (PSG) isolated from from midguts of infected sand flies (specific vector of *L. tropica*, *Phlebotomus sergenti*) as described in (Rogers et al. 2004), where this approach has been used for mouse infection with *L. mexicana* co-inoculated with PGS of *Lutzomyia longipalpis*. As described for other sand flies, PSG enhanced lesion development induced by *L. tropica* more than saliva and this procedure has been used for further analysis (data not shown).

Genetic differences in skin pathology caused by infection with *L. tropica*

We infected both females and males of the strains BALB/c, STS and RC strains CcS-3, CcS-5, CcS-11, CcS-12, CcS-16, CcS-18 and CcS-20 to study susceptibility to *L. tropica*. The skin pathology progressed slowly and started as nodules at the site of *L. tropica* infection appearing between week 11 and 20, which transformed in susceptible strains into skin lesions (Figure 1). Females of strains CcS-16, BALB/c, CcS-11 and CcS-20 were relatively susceptible to the infection and developed skin lesions after week 18, larger lesions being observed in CcS-16 (Figure 2A). Females of strain CcS-16 exhibited skin lesions from week 18 till the end of experiment (week 43). In females of the strains BALB/c, CcS-11 and CcS-20 the lesions partly healed and tended to transform back to nodules after week 30. In females of strain CcS-11, small skin nodules appeared at week 14, and most males died before week

18, females died later (at 32-42 weeks of infection in 42-week experiment, and only one female died at week 13 in 21-week experiment). Males of strain CcS-16 developed small lesions that later healed, whereas males of BALB/c developed small lesions after week 30 (Figure 2B).

Strains CcS-12 and CcS-18 are intermediate in susceptibility to skin pathology. Both females and males of the strain CcS-12 developed small skin lesions at the late stages of infection (after week 37). CcS-18 females developed nodules or small lesion that healed, whereas males developed only small nodules.

Strains STS, CcS-3 and CcS-5 were resistant to development of skin lesions. Both females and males of the strain CcS-3 had small skin nodules at the late stages of infection and did not develop skin lesions till the end of experiment. Females of the strains STS and CcS-5 were resistant to *L. tropica*, and few of them developed small nodules at the site of infection. Males of the strains STS and CcS-5 exhibited skin nodules which started to develop at week 35 and persisted till the end of experiment at week 43.

Sex has different effect on skin pathology caused by *L. tropica* and *L. major*

Sex differences observed in susceptibility to *L. tropica* led us to ask whether similar influence takes place also in susceptibility to *L. major*. As our previous research with *L. major* were focused on analysis of females (Lipoldová et al. 2002), in this study we have infected with *L. major* both females and males of strains BALB/c, STS and RC strains CcS-3, -5, -11, -12, -16, -18 and -20. Both males and females of all analyzed strains developed larger skin lesions after infection with *L. major* (Table 1) than when infected with *L. tropica*. The effect of sex was different in experiments with *L. major* and *L. tropica*. After the infection with *L. tropica*, females of CcS/Dem strains in most cases exhibited more extensive skin pathology than males (Figure 2 A, B), whereas after infection with *L. major*, skin lesions in males and females of strains BALB/c, STS, CcS-11, CcS-12, CcS-16 and CcS-20 did not differed,

whereas males of strains CcS-3 (P = 0.001), CcS-5 (P = 0.001) and CcS-18 (P = 0.043) developed larger skin lesions than females.

Draining lymph nodes of all tested strains contained viable parasites.

In vitro culture tests showed that all tested mice, including strains that did not exhibit any skin pathology, contained viable parasites in their inguinal lymph nodes both 21 and 43 weeks after infection. (Figure 3-5). None of the strains contained more parasites in the lymph nodes than the background parental strain BALB/c (Figure 4, 5). At week 21 after infection, females of the strains STS and CcS-5, and males of the strain CcS-5 contained significantly lower parasite numbers than the strain BALB/c (P = 0.0002 for females and P = 0.0093 for males). At week 43 after infection, females of the strains STS, CcS-5 and CcS-11, and STS and CcS-11 males differed from BALB/c (P = 0.00000001 for females and P = 0.0004 for males). At week 43, females of strain CcS-18 had higher parasite count than males (P = 0.0209), whereas males of strain CcS-5 had higher parasite load than females (P = 0.0143). In both experiments counted together, females of CcS-18 (P = 0.0318) strain had higher parasite load than males, whereas STS males had higher parasite numbers than females (0.0097) (Figure 4).

***L. tropica* can invade spleens of several infected mouse strains**

We did not observe any splenomegaly and hepatomegaly induced by infection with *L. tropica*. However, *in vitro* cultures have shown that 21 weeks after infection 50%, 66.7% and 16.7% spleens of female mice of strains CcS-3, -18 and -20, respectively, contained viable parasites. We were also able to cultivate parasites from 16.7% and 33.3% of spleens of males of strains BALB/c and CcS-20, respectively. Parasite presence in spleens was confirmed by histological examination (Figure 6). Parasite numbers in spleens of other strains were either below the level of detection or absent.

Systemic reactions after infection with *L. tropica*

We measured levels of IL-4, IL-12, IFNγ, GM-CSF (granulocyte-macrophage colony-stimulating factor), CCL2 (chemokine ligand 2) /MCP-1 (monocyte chemotactic protein-1), CCL3/MIP-1α (macrophage inflammatory protein-1α), CCL4/MIP-1β (macrophage inflammatory protein-1β), CCL5/RANTES (regulated upon activation, normal T-cell expressed, and secreted) and CCL7/MCP-3 (monocyte chemotactic protein-3) and IgE in serum of uninfected and infected mice.

No significant difference was found in IL-4, IL-12, IgE, IFNγ and GM-CSF levels in serum of infected mice in comparison with noninfected controls (data not shown). We have observed increase of serum levels of CCL2, CCL3 and CCL5 in strains BALB/c, CcS-11, CcS-20, and CcS-18 (Figure 7). Peak of increase of levels of these chemokines usually followed start of lesion development. Increase were more expressed in females than in males. Females of strain CcS-16 exhibited an unique pattern of kinetics of CCL3 and CCL5 levels, which differed from all other strains (Figure 7) and also from CcS-16 males (Figure 8). We observed two peaks of increase of serum levels of CCL3 and CCL5 in females of CcS-16; one before the development of skin lesions, the other after the decrease of lesion size, and there were almost no changes in CCL2 level. CcS-16 males had slight increase of CCL3, CCL5 and CCL2 (Figure 8), kinetics of increase being similar as those in females and males of other strains.

Discussion

Similarly as in experiments described by others (Lira et al. 1998), the disease progress was slow. Comparatively to the results in our previous experiments with *L. major* infection (Lipoldová et al. 2000, Havelková et al. 2006), the disease manifestations were less progressed in *L. tropica* infected mice. The nodules at the site of infection appeared in

different strains after the 11-th week of infection, and only some of the tested CcS/Dem strains developed skin lesions. Similarly as in *L. major* infection, STS and CcS-5 strains showed the highest resistance to *L. tropica* induced disease.

Different genetic influence on susceptibility to *L. tropica* and *L. major*

In our previous experiments with *L. major*, females of the strains BALB/c, CcS-12, -16 and -18 were susceptible to development of skin lesions, whereas strains STS and CcS-5 were resistant, and CcS-3, -11, and -20 were intermediate (Lipoldová et al. 2002).

In general, mice infected with *L. tropica* exhibited less intense skin pathology than mice infected with *L. major*.

Both in experiments with *L. tropica* and *L. major* the impact of sex on the disease development was observed, however the influence of sex was different. In *L. major* infection, males of all strains exhibited more severe pathological changes than females, but in *L. tropica* experiment the situation tended to be opposite. Females of most strains had more extensive skin pathology. Moreover, sex-dependent differences were also found in other disease manifestations: parasite load in draining lymph nodes, chemokine levels in serum and correlations between chemokine levels and skin lesion development. This fact suggests that some genes controlling susceptibility to *L. tropica* might be sex dependent. Studies of leishmaniasis caused by *Leishmania tropica* in human population of some geographic regions also showed higher persistence of the parasite in women (Greenblatt 1980).

Females of the strain CcS-16 exhibited the highest level of skin pathology and developed large skin lesions at 16-th week of infection (Figures 1, 2), exceeding skin manifestations in BALB/c susceptible mice. Similar results were described for *L. major* infection studies when part of the genome of resistant STS strain, represented in CcS-16 mice, contained genes responsible for even higher susceptibility of CcS-16 mice than BALB/c (Lee et al. 1995, Roberts et al. 1997, Vladimirov et al. 2003).

As well as previous *L. major* experiments (Vladimirov et al. 2003), our present *L. tropica* studies confirmed that extent of the pathological changes in different organs did not directly correlate with parasite load. On the background of relatively low symptoms, we found viable parasites in inguinal lymph nodes of all tested strains (Figure 4, 5). In some strains (CcS-3, -18, 20 females, and males CcS-20 and BALB/c males), we also observed visceralization of pathology with viable parasites in spleen (Figure 6). These data indicate that parasite spread to the different organs and other manifestations of the disease are dependent on the genome of the host.

The role of CC-chemokines CCL2, CCL3, CCL4 and CCL5 was previously tested in experiments with *L. donovani* and *L. major*. CCL3, CCL4 and CCL5 chemokines are known to be produced by macrophages in response to *L. donovani* infection (Dasgupta et al. 2003).

After infection the strains BALB/c, CcS-11, CcS-18, CcS-16 and CcS-20 have increased levels of chemokines CCL2, CCL3 and CCL5 in serum. Unexpectedly and in contrast with the other strains tested, the CcS-16 females but not males exhibited a unique pattern of this systemic reaction, characterized by an additional early peak of chemokine levels. It suggests that these early peaks of CCL3 and CCL5 (Figure 8) might be associated with an increased susceptibility of CcS-16 females to *L. tropica*.

In previous studies with experimental *L. major* infection, CCL5 contributed to host resistance. CCL2 alone did not correlate with resistance (Santiago et al. 2004), however, together with CCL3, it significantly enhanced parasite killing in *L. infantum* infected human macrophages (Brandonisio et al. 2002). It is also known that CCL2 and CCL3 stimulate antileishmanial response via the induction of NO-mediated regulatory mechanisms to control the intracellular growth and multiplication of *L. donovani*. Expression of iNOS mRNA was induced markedly in the infected macrophages preincubated with CCL2 and CCL3 (Battacharyya et al. 2002). In human, CCL2 contributed to healing, but CCL3 was associated with chronic lesions during *L. mexicana* infection (Ritter et al. 2002). These studies of the role

of different C-C chemokines in leishmaniasis revealed that a coordinated interaction of several chemokines is extremely important for successful immune response against *Leishmania*.

Chemokines have different roles in *Leishmania* infection. On the one hand, they recruit immune cells to the site of parasite delivery, take part in adaptive response, macrophage activation and parasite killing (Teixeira et al. 2006). On the other hand, chemokines influence *Leishmania*, involving the inner regulatory mechanisms of parasitic cells.

Chemokines are proteins that specifically bind to certain transmembrane G-protein coupled receptors (Baggiolini et al. 1997). *Leishmania* parasites express receptor-like molecules on their surface that can bind certain specific host derived chemokines with high affinity. It was shown that CCL3, CCL4 and CCL5 can chemoattract parasites to the source of the chemokine secretion and these molecules compete for the same binding site on the parasite membrane. The binding of chemokines to the promastigote surface was proved to be functional and probably G-protein mediated. Presence of CCL3, CCL4 and CCL5 caused Ca^{2+} mobilization in parasitic cells that was almost completely abrogated by pertussis toxin, a G-protein blocker, similar to that observed in the mammalian host (Roychoudhury et al. 2006). Previous studies showed that changes in cytosolic Ca^{2+} homeostasis in parasitic cells lead to collapse of mitochondria membrane potential with following death of parasites via apoptosis (Mukherjee et al. 2002). In this way, production of chemokines in response to *Leishmania* infection may facilitate killing of parasites.

It is also necessary to mention that apoptosis may be beneficial for the parasite itself. Apoptotic cells provide signals that could enhance survival of the entire population of *Leishmania*. It was shown that exposure to phosphatidylserine (an early hallmark of apoptosis) on the surface of *L. amazonensis* amastigotes drives internalization and establishment of the parasite in host cell (Wanderley et al. 2005, Wanderley et al. 2006).

Though the exact mechanism by which phosphatidylserine exposure increases amastigotes infectivity is not fully understood, a model for the mechanisms of parasite survival was postulated on the background of previous research with *L. infantum*, *L. major* and *Trypanosoma cruzi*. It is known that activated macrophages metabolize L-arginine via two main pathways. Nitric oxide synthase (iNOS) pathway leads to nitric oxide (NO) generation that inhibits replication of parasites, whereas arginase pathway results in polyamine synthesis stimulating replication of parasites inside host cells. Recognition of phosphatidylserine on the surface of amastigotes leads to TGF-β release by infected macrophages. This cytokine then stimulates the arginase activity of infected and surrounding cells, consequently increasing polyamine synthesis and stimulating survival and replication of parasites. Thus, parasites, displaying apoptotic phenotype, may be essential for persistence of parasitic population in the host organism (Wanderley et al. 2005).

Immune response to parasite invasion and control of the pathogen dissemination appears to be a very complicated process with preferable activation of different genes and complexes of genes depending on the host genotype. During wide studies of *L. major* infection, more than 25 *Leishmania* major response loci (*Lmr*) and range of genes were shown to contribute to the course of the disease (Beebe et al. 1997, Roberts et al. 1997, Lipoldová et al. 2000, Badalová et al. 2002, Vladimirov et al. 2003, Havelková et al. 2006, Kurey et al. 2009). Variety of effects of the particular *Lmr* may be due to multiple effects of one or several genes. Our present study disclosed similar trends in leishmaniasis caused by *L. tropica*.

In our experiments, many of RC strains and exhibited different susceptibility to *L. major* and *L. tropica*. This fact demonstrates existence of species specific controlling host genes with different functions. Therefore without combining the two components of genetic variation involved in the outcome of *Leishmania* infection - variation of the host and variation of the parasite - the understanding of the mechanisms of disease will remain incomplete.

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Figures

Figure 1. Skin lesion caused by *Leishmania tropica* in female of CcS-16 strain at 43 week after infection.

Figure 2. Kinetics of skin lesion development of CcS/Dem strains after infection with *L. tropica*.

Individual strains are marked with different colors. The columns show median values of lesion size in females (A) and males (B). After the infection with *L. tropica*, females of the strains BALB/c, STS, CcS-3, CcS-12, CcS-16, CcS-18, CcS-20 in most cases exhibited more extensive skin pathology than males.

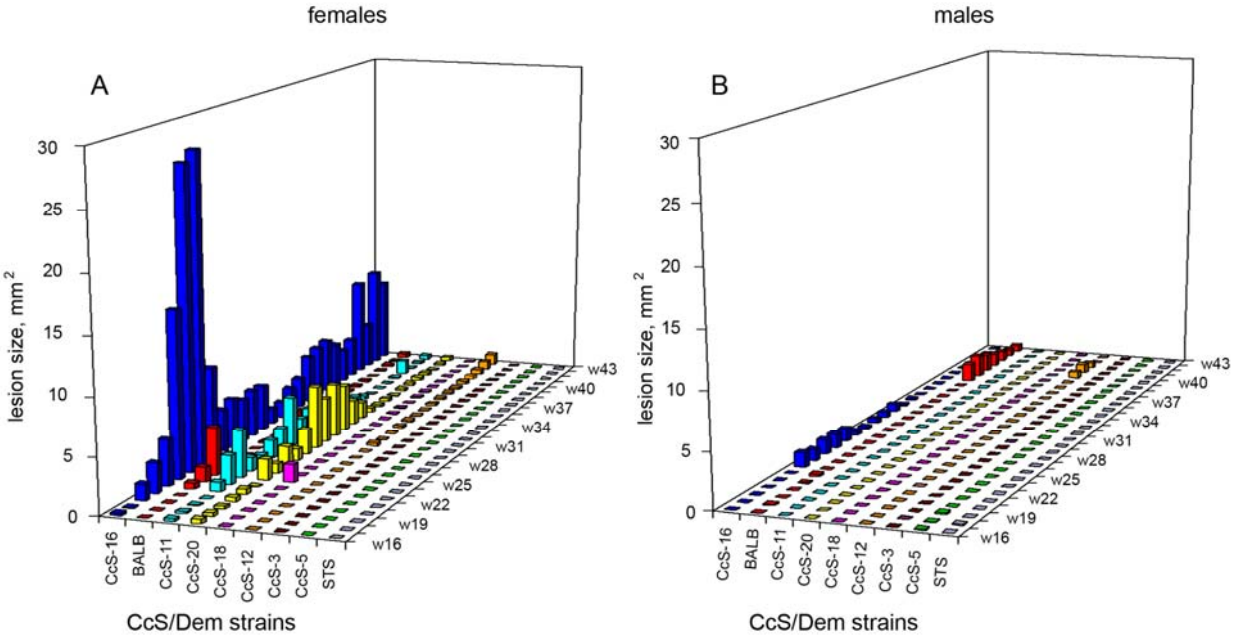


Figure 3. *Leishmania tropica* parasites inside the macrophage.

A smear of the inguinal lymph node of BALB/c male was stained with the anti-*Leishmania* lipophosphoglycan monoclonal antibody (CLP003A, Cedarlane, Hornby, Canada) and TRITC labeled IgM (115-025-020, Jackson Immunoresearch, West Grove, PA, United States of America), all diluted 1:100. Nuclei of the cells were stained with DAPI. Magnification × 1000. Parasites are shown with arrows.

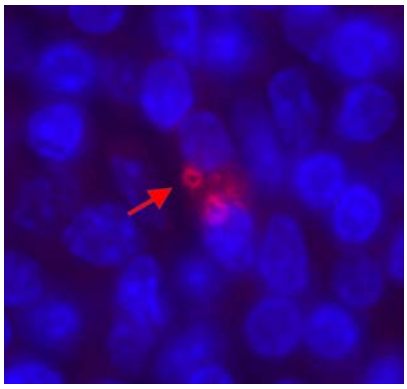
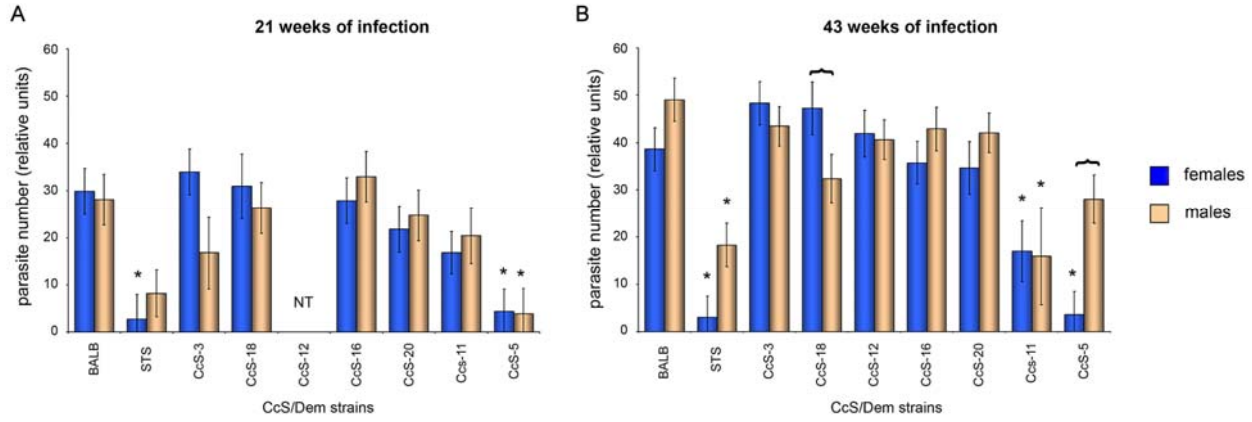


Figure 4. Number of parasites cultivated from lymph nodes of mouse strains 21 and 43 weeks after infection. Asterisks show strains that exhibited parasite load significantly different from BALB/c. Brackets indicate strains with differences between males and females.



Week 21 after infection (A). Females of the strains STS and CcS-5, and males of the strain CcS-5 contained lower parasite numbers than BALB/c (P = 0.0002 for females and P = 0.0093 for males). Week 43 after infection (B). STS, CcS-5 and CcS-11 females, STS and CcS-11 males differed from BALB/c (P = 0.00000001 for females and P = 0.0004 for males). Females of strain CcS-18 had higher parasite count than males (P = 0.0209), males of strain CcS-5 had higher parasite count than females (P = 0.0143). In both experiments counted together, females of CcS-18 strain had higher parasite load than males (P = 0.0318), whereas STS males had higher parasite numbers than females (0.0097).

Figure 5. Parasites in hematoxylin-eosin stained lymph node smears.

All tested mice contained viable parasites in their inguinal lymph nodes. Infected BALB/c female (A); noninfected control BALB/c female (B); infected BALB/c male (C); noninfected control BALB/c male (D); infected STS female (E); noninfected control STS female (F); infected STS male (G); noninfected control STS male (H). Parasites are shown with arrows.

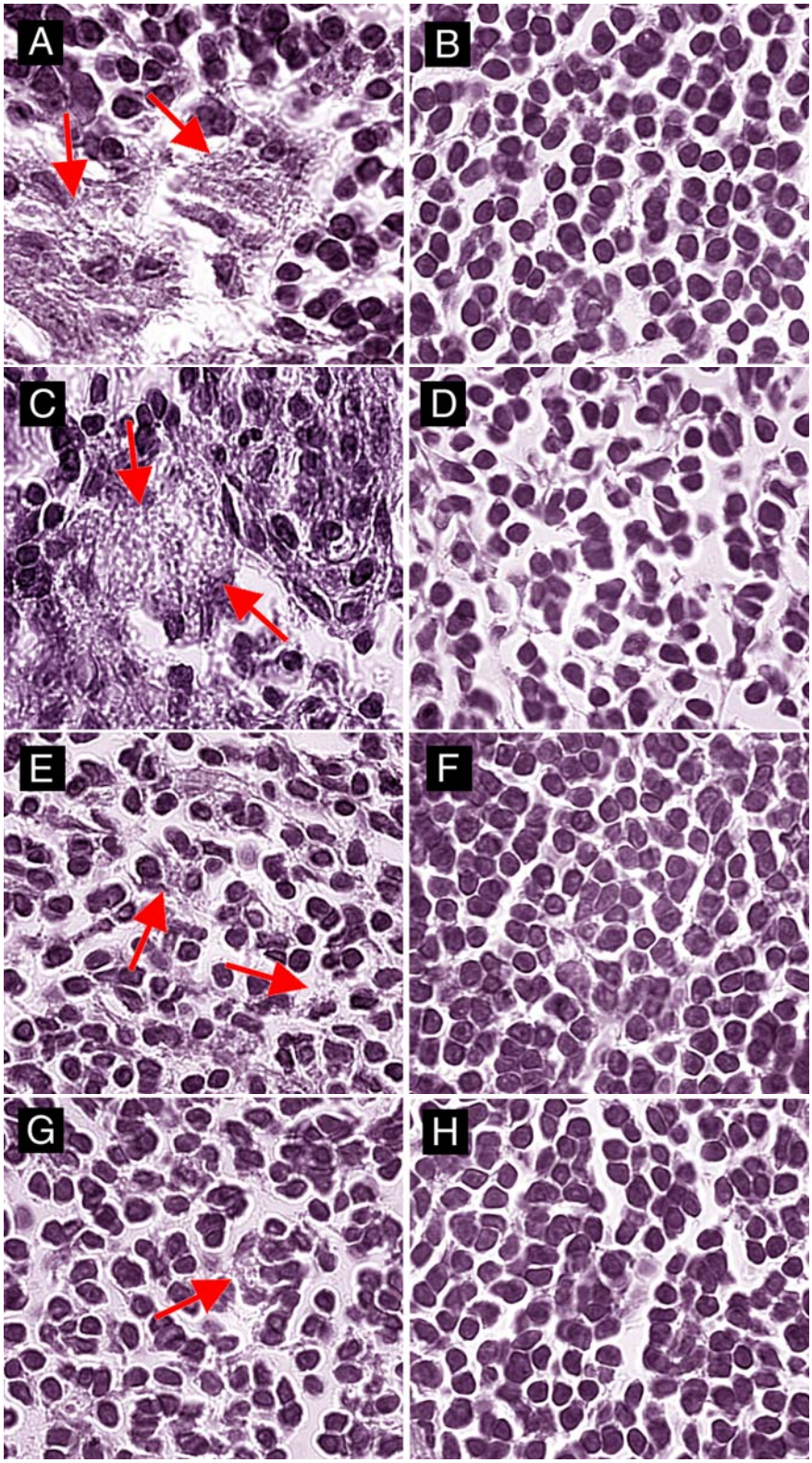


Figure 6. Parasites in hematoxylin-eosin stained spleen smears.

L. tropica can invade spleens. Arrows show a group of parasites among spleen cells in infected BALB/c male (A); noninfected control BALB/c male without parasites (B).

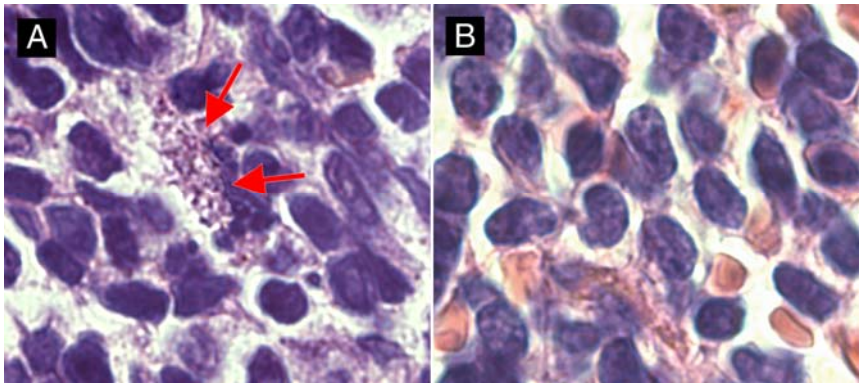


Figure 7. Relationship between chemokine expression and lesion size development during the course of *L. tropica* infection.

We compare kinetics of skin lesion development and serum levels of CCL2, CCL3 and CCL5 in females.

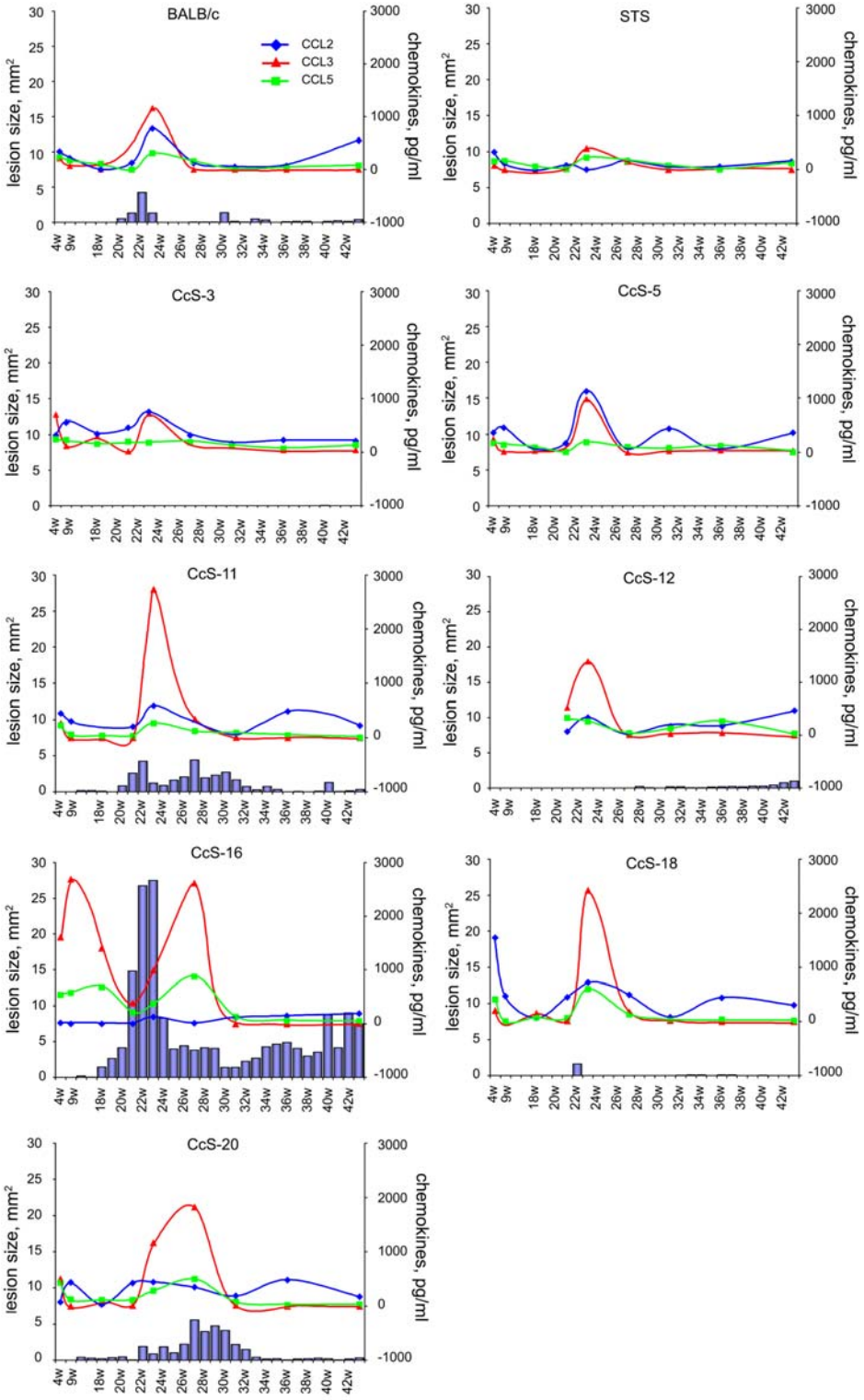


Figure 8. Different kinetics of chemokine expression in females and males of CcS-16 strain. CcS-16 males revealed remarkably lower peaks of chemokine concentration than in females. Kinetics of CCL3 levels in serum of BALB/c (A) and CcS-16 (B) and serum levels of CCL5 in BALB/c (C) and CcS-16 (D) mice is shown.

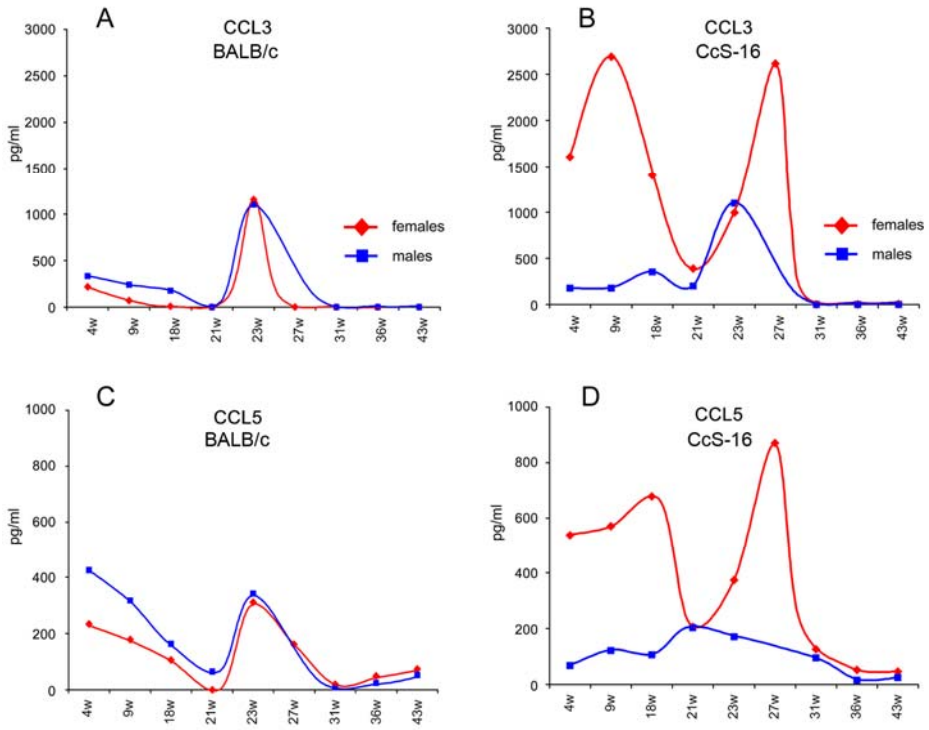


Table 1. Sex differences in lesion size (week 8) after *L. major* infection

Strain	Sex	Number of mice	Lesion size week 8	*P*-level of difference between females and males
			mean ±SD	
BALB/c	females	27	116.7 ±30.4	0.098
	males	30	144.3 ±60.5	
STS	females	8	3.0 ±7.4	0.252
	males	10	6.9 ±8.2	
CcS-1	females	15	124.3 ±28.9	**0.041**
	males	17	147.1 ±46.6	
CcS-3	females	10	45.2 ±13.5	**0.001**
	males	10	73.8 ±17.2	
CcS-4	females	13	81.4 ±36.9	0.953
	males	12	81.6 ±41.8	
CcS-5	females	26	14.9 ±15.3	**0.001**
	males	32	29.7 ±27.9	
CcS-7	females	15	103.4 ±38.5	0.175
	males	16	126.7 ±49.0	
CcS-9	females	21	133.7 ±49.5	0.579
	males	13	149.7 ±56,7	
CcS-11	females	20	61.7 ±38.1	0.074
	males	21	68.6 ±44.2	
CcS-12	females	18	134.6 ±27.0	0.296
	males	17	146.5 ±44.4	
CcS-15	females	11	106.4 ±35.0	0.433
	males	14	104.2 ±53.3	
CcS-16	females	11	138.0 ±32.7	0.105
	males	13	160.8 ±28.2	
CcS-18	females	9	97.5 ±36.8	**0.043**
	males	4	126.2 ±38.0	
CcS-20	females	14	51.6 ±31.7	0.665
	males	18	57.4 ±36.0	

Discussion and perspectives

Detection and quantification of different parasites belongs to the hot topics of the tropical medicine. *Leishmania* is among the first targets on the list of interest. The present work proves how different approaches can be used to obtain valuable scientific information. The experiments included to this thesis estimated various disease manifestations, such as lesion size; parasite load in spleen, lymph nodes and infected ears; levels of cytokines, chemokines and immunoglobulins; splenomegaly and hepatomegaly. Full detailed discussion, involving all obtained results, is given in each publication or manuscript which can be found in the "Results" section. Here the attention will be predominantly concentrated on the data concerning importance of the parasite load parameter in the *Leishmania* research. However, all other results could not be omitted since leishmaniasis is a complex disease and it is absolutely necessary to have in mind a complete overview of the question.

Summarizing the information about the wide range of existing assays for measurement of the *Leishmania* parasite load, it is possible to select the most optimal method which would be suitable for certain study. The method of choice always depends on the several main criteria. At the first step, the clinical form of leishmaniasis (cutaneous, mucocutaneous or visceral) determines types of samples that are usually available for analysis (biopsies, aspirates, blood etc.). Second, purposes of the study influence the selection of methods for the analysis. It should be taken into account, whether the simple detection of the genus *Leishmania* is sufficient or quantification of parasite load would be beneficial; or whether it is necessary to identify parasite species. Third, the amount of financial support and equipment of the laboratory significantly narrows the spectrum of available techniques. Last but not least, the presence of trained personnel can play an important role (for example, microscopy analysis requires participation of the experienced specialist). Research work described in this

thesis takes into account advantages and disadvantages of the methods and illustrates their successful application.

I. Development and optimization of the methods

Reading the "Introduction" with a plenty of the techniques, a question possibly arises: if there are so many, why we developed another one? Let's take a closer look into the details of the assays that were designed, optimized and used in our laboratory to understand their particular importance. Despite of the abundance of the methods for measurement of the parasite burden, most of them are problematic to use if more than one hundred samples has to be tested fast, in one run. In addition, many of them have significant drawbacks.

As mentioned above, we were first to introduce two labeled primers in PCR-ELISA developed for leishmaniasis research. All other similar methods include only one labeled primer and label the product with an additional probe before the ELISA step. On one hand, it can help to increase specificity of the detection, if the primers that are used in PCR are not specific enough or conditions of the reaction are not properly balanced. On the other hand, each additional step in the reaction itself increases the possibility of a mistake. In a case of non-complete or non-specific binding of the labeled probe to the PCR product, it is impossible to detect the presence of the technical mistake, because there is no method to find out if such hybridization was absolutely successfull. As a consequence, it increases the possibility to obtain false positive, as well as false negative, results. Such discrepancies may play especially dramatic role during examination of clinical samples. When two primers are labeled in advance and the conditions of PCR are carefully optimized, the method can give a guaranteed result. A particular advantage is the ability to make an additional control procedure and check the PCR product by agarose gel electrophoresis (see Kobets et al. 2010) to ensure that the synthesis was specific and the result is reliable. Besides the potential future perspectives of the application for clinical diagnostics, this modification of PCR-ELISA has

been already successfully used for detection of the genetic loci that control parasite dissemination (Kurey et al. 2009), as well as for research of vector saliva influence on the development of leishmaniasis (Rohoušová et al. 2011).

It is worth to stress that primers specific for kDNA of *Leishmania* showed higher sensitivity than primers that target other regions (Nicolas et al. 2002, Bensoussan et al. 2006, Tupperwar et al. 2008, Roelfsema et al. 2010). PCR using such kind of primers was recommended to use as a gold standard after several independent comparative studies (Bensoussan et al. 2006, Nasereddin et al. 2008, de Lima et al. 2010).

The second method that was modified and applied in our laboratory was a protocol for measurement of parasite load using tissue cultures. This method is based on the limiting dilution assay (Titus et al. 1985, Lima et al. 1997) and targets only viable parasites that are able to multiply. This specific feature makes cell culture based techniques still useful for various purposes, such as antileishmanial drugs development, in which distinction between dead and alive parasites is important. The most significant change introduced to the method was cultivation of cells in only one concentration, chosen according to the results of the previous experiments. This modification brought a big advance and allowed testing of more than a hundred samples in one experiment, what would be extremely laborious and technically very difficult with classical limiting dilution assay (Kobets et al., in preparation).

II. *Leishmania* and different host genotypes

As described above, genetically different mice from the experiments with *Leishmania major* (BALB/c, CcS-11 and F_2 hybrids between these strains) were tested for parasite load with modified PCR-ELISA protocol. Parasite presence in secondary lymphoid organs is controlled by two loci, *Lmr20* and *Lmr5* (Kurey et al. 2009, table 3). In addition to parasite load in draining lymph nodes, *Lmr20* influenced levels of serum IgE and IFNγ; whereas *Lmr5*

was also involved in control of lesion size, parasite load in spleen, splenomegaly, serum IgE, serum IFNγ, and serum IL-4 (Kurey et al. 2009, table 2).

On the basis of the obtained results, we can conclude that parasite numbers in lymph nodes and in spleen are controlled independently. *Lmr20* is not involved in regulation of skin lesion size, splenomegaly, or hepatomegaly, but it influences systemic immunological changes along with parasite numbers in lymph nodes. Control of spread of parasites to the spleen is exercised by another locus - *Lmr5* along with pathology of another organ (the skin lesions) and systemic immune responses. In both *Lmr* loci, the BALB/c allele is associated with more severe pathology. There was also a strong sex effect on parasite numbers in draining lymph nodes and spleen. Males showed higher susceptibility to *L. major* and developed more severe pathology than females (Kurey et al. 2009).

In leishmaniasis research, *Lmr20* on the chromosome 1 and *Lmr5* on the chromosome 16 were the first genetic loci that control parasite dissemination in *Leishmania major* infection detected in genome-wide study.

The importance of involvement of *Leishmania tropica* in human leishmaniasis has been recognized only recently. Similarly as *L. major, L. tropica* causes cutaneous leishmaniasis in humans, but was also reported to visceralize and cause systemic illness (Magill et al. 1993, Alborzi et al. 2008, Weiss et al. 2009). Genetic basis of susceptibility or resistance to *L. tropica* infection has been poorly studied because of the absence of the suitable animal models.

Similarly to the experiments of other groups (Lira et al. 1998), the disease progress was very slow. In comparison with the results of our previous studies with *L. major* infection in RC strains (Lipoldová et al. 2000, Lipoldová et al. 2002, Havelková et al. 2006), all disease manifestations were less progressed in *L. tropica* infected mice. Moreover, genotype effects in RC strains were different after *L. tropica* and *L. major* infection (Kobets et al., in preparation).

Parasite load in the inguinal lymph nodes did not correlate with development of skin lesions after *L. tropica* infection. Such existence indicates independent genetic control of different disease manifestation is similar to the experiment with *L. major* infected F_2 hybrids between BALB/c and CcS-11, described above (Kurey et al. 2009). Microscopy examinations of histological sections from the spleen confirmed the presence of parasites, previously detected with culture method. Thus, we first report visceralization of *L. tropica* in mice. Taking into account the information that *L. tropica* was able to cause viscerotropic leishmaniasis in human (Magill et al. 1993, Alborzi et al. 2008, Weiss et al. 2009), the mouse model of visceral pathology could contribute to understanding of genetical background of this phenomenon. In the *L. tropica* experiment parasite load in spleens and inguinal lymph nodes was measured with tissue culture method. Unfortunately, it was not possible to compare this result with quantification of parasites in samples from the same mice using PCR-ELISA, because the experiment was performed at the time when the PCR-ELISA method has not been yet optimized.

The animal model of leishmaniasis using CcS strains proved to be a powerful tool to characterize the mechanisms of the host-parasite interactions. Our studies show that comparison of *L. tropica* and *L. major* infections indicate existence of the species-specific patterns of response to the certain pathogen, with different sex effects and different host susceptibility genes (Kobets et al., in preparation). In human population, the influence of genotype (Lipoldová and Demant 2006, Sakthianandeswaren et al. 2009) and sex on response to different parasite infections was also observed. It was shown that women tourists more rarely get vector-borne diseases, including leishmaniasis (Shlagenhauf et al. 2010). In contrast, no sex differences were found in the studies of patients with visceral leishmaniasis from Sudan (El-Safi et al. 2002) and cutaneous leishmaniasis from Afghanistan (Reithinger et al. 2010). However, in the endemic areas of Brazil and Colombia, males were reported to be more susceptible to *L. braziliensis* (Jones et al. 1987, Weigle et al. 1993). The data, coming

from different populations, varied. This fact illustrates the complex influence of genetic and environmental factors. There is relatively few data derived from human studies due to the general complications of human genetic research. However, such information is remarkably valuable and may confirm the theory that leishmaniasis in heterogeneous human population is influenced by multiple factors, such as genotype of the host, sex and *Leishmania* species. As mentioned above, environmental and social conditions also play a significant role in susceptibility to leishmaniasis.

III. Immunization with sand fly saliva

Sand flies are bloodsucking insects that are natural vectors of *Leishmania* parasites. They deposit parasites into the host skin along with saliva. There is lack of human studies that would reflect complex effects of sand fly saliva; however, it was shown that individuals exposed to sand fly bites develop specific immune response to salivary proteins (Rohoušová and Volf 2006). In the endemic areas, people are exposed to bites of uninfected sand flies long term and may also experience sand-fly free periods. It was hypothesized that the enhancing effect on *Leishmania* infection could be neutralized in humans vaccinated against vector saliva, thereby preventing the establishment of infection. This concept was proven in various animal models, though such level of protection has not been reported from the field. Despite of continuous exposure of the individuals to the insect bites, leishmaniasis still persists in the endemic areas (Rohoušová et al. 2011).

In our present study, *Leishmania* infection was monitored in BALB/c mice exposed to *Phlebotomus duboscqi* bites following four different schemes, mimicking short- and long-term exposure to the saliva during different sand fly seasons (Rohoušová et al. 2011, fig. 1A). In the group of mice that got 2 days exposure to sand fly bites right before the infection (group 2), the cumulative parasite load in immunized mice was significantly reduced, as well as lesion development. Moreover, only the protected group (group 2) had significantly lower

anti-*L. major* IgG levels compared with non-immunized mice (Rohoušová et al. 2011, fig. 1F), correlating with the lowest parasite load in this group. The protective effect was lost in the group of mice that got the same short exposure but 15 days before the infection (group 2+0), as well as in both groups immunized during long period (groups 15 and 15+0). We suggested that long-term immunization by sand fly bites might cause possible desensitization to salivary antigens, but such groups (15 and 15+0) also showed absence of significant protective effects against *Leishmania*. We can hypothesize that long-term immunization tends to skew the immune system towards a Th2 response which could be associated with the lack of protection. Additionally, the severity of skin damage could be due to the deposition of immune complexes in the skin, leading to an exacerbated immune response from the early stage of lesion development. These immune complexes are more likely to form between anti-saliva antibodies and salivary proteins since no binding was observed between anti-*P. duboscqi* saliva antibodies and *L. major* promastigotes (Rohoušová et al. 2011). On the other hand, no correlation was found between anti-*P. duboscqi* IgG and parasite load in the draining lymph node, indicating that preexposure to *P. duboscqi* bites could completely prevent parasite dissemination, though higher parasite load in the ears indicate that in a bitten hosts *L. major* preferentially multiplies in the skin tissue.

Our experiments proved that the immune response resulting from this immunization scheme have certain protective effects, however the history of exposure may significantly change the character of anti-saliva immune response with substantial consequences for *Leishmania* infection development. This assumption is in agreement with field results from an endemic area of cutaneous leishmaniasis caused by *Leishmania braziliensis* (de Moura et al. 2007, Rohoušová et al. 2011).

This is one more study in which we used PCR-ELISA (Kobets et al. 2010). Measurement of the parasite load helped to reveal different aspects of the influence of sand fly saliva on the development of leishmaniasis. Our results might help to explain the

persistence of *Leishmania* infection in endemic areas and should be taken into account when designing and testing vaccines based on vector salivary proteins (Rohoušová et al. 2011).

IV. Microscopy as a visual proof

It was mentioned in the "Introduction" chapter that measurement of parasite burden using microscopy assays has been a classical standard for *Leishmania* detection and quantification from the beginnings of leishmaniasis research until recently. It is also stated that studies performed during the last years proved the kDNA specific PCR to be a gold standard, and also proposed that novel techniques should be compared with it for validation (Bensoussan et al. 2006, Nasereddin et al. 2008, de Lima et al. 2010). However, this new advance should not set various microscopy assays aside from the use. Practice still shows that detection of *Leishmania* parasites both with light and fluorescent microscopy remains an informative and irreplaceable tool for visual illustration of the experimental results. Using histological examination, we confirmed presence of parasites in inguinal lymph nodes in *L. major* study (Kurey at al. 2009) and proved the fact of penetration of the parasite into the spleen in *L. tropica* project (Kobets et al., in preparation). A classical method of parasite count in tissue smears was applied for validation of the improved PCR-ELISA (Kobets et al. 2010).

V. Perspectives of leishmaniasis research

More than 20 *Leishmania major* response loci (*Lmr*) were discovered in our laboratory using a RCS mouse model and infection with *Leishmania major*. Two of these loci, described in the present work, influence dissemination of parasites to the organs of the infected F_2 hybrids (Kurey et al. 2009). These loci were detected with our PCR-ELISA protocol. In our planned studies, the method will be used for measurement of parasite load in F_2 hybrids prepared from other RCS to find other *Lmr* loci that are involved in control of *Leishmania* in

the infected organism. Though the relatively large length of *Lmr* loci does not allow fast detection of the individual genes involved into the disease development, the step forward can be done in different ways. First, preparation and analysis of mouse recombinants, containing a certain locus separated into several short segments, can narrow the area of interested for further studies. Second, examination of the expression profiles of genes using microarrays and RT-PCR may reveal variations between tested strains and point the possible candidate genes. In addition, studies concerning functional analysis of the key molecules that play role in host-parasite interactions during leishmaniasis (such as iNOS, arginase, NADPH, Fas, FasL, perforin) can contribute to the research of the leishmaniasis.

In *Leishmania tropica* research, we detected that infected females of the strain CcS-16 appeared to be the most susceptible (Kobets et al., in preparation). As a consequence, this strain became of high interest for us. Experiments using F_2 hybrids between BALB/c and CcS-16 may reveal effects of parasite control (as well as other disease parameters) specific for *L. tropica* infection. PCR-ELISA, originally developed for *L. major* detection, is also suitable for measurement of *L. tropica*.

The present work shows the importance of parasite detection and quantification as a parameter that can significantly contribute to understanding of the mechanism of leishmaniasis. After design of a suitable primer specific for the target organism, the improved method of PCR-ELISA can be applied for quantitative estimation of the infection with other pathogens (for example, *Trypanosoma*, another parasite studied in our laboratory).

Conclusions

I. We designed and optimized two assays for detection and quantification of *Leishmania* parasites and used classical ones for validation.

1. The improved PCR-ELISA method uses two labeled primers and allows measurement of parasite load in tissues of the infected organism with high specificity and sensitivity.

2. Modified culture-based method enables processing of much larger numbers of samples than classical limiting dilution assay and detects viable parasites in tissue samples.

3. Using of microscopy assays served as a classical standard for validation of novel methods, as well as illustrated the obtained results.

II. Measurement of parasite load revealed important aspects of the disease development, and showed genotype-dependent variations of the immune response during leishmaniasis. Estimation of parasite numbers also helped to characterize effects of sand fly saliva on the pathological changes in the infected organism.

1. Two novel loci that control parasite load were first detected in genome-wide study.

2. Parasite dissemination to the spleen and lymph nodes is controlled independently by the genetic loci situated on two different chromosomes.

3. We first established the mouse model of *Leishmania tropica* infection using RC strains.

4. In *L. tropica* infection, parasite numbers were much lower in comparison with *L. major* experiments. This data confirms the fact that, in addition to genetic and environmental factors, immune response of the organism strongly depends on the *Leishmania* species.

5. *L. tropica* parasites were present in lymph nodes of all tested strains, and some strains penetrated to the spleen; however, no splenomegaly was observed. Parasite load also did not correlate with skin lesion that suggests independent genetic regulation of these disease parameters.

6. Measurement of the parasite load helped to reveal various types of the influence of sand fly saliva on development of leishmaniasis. Protective effect of saliva is limited and depends on the history of exposure to the insect bites.

7. Immunization with sand fly saliva does not completely prevent dissemination of *Leishmania major* parasite to lymph nodes; however it can reduce pathology in case of short-term exposure before the infection. This data should be taken into account during development of saliva-based vaccines.

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Curriculum vitae

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Degrees

2002 – **BSc** (Biochemistry, Graduated with Honors): Karazin Kharkiv National University, Kharkiv, Ukraine

2003 – **MSc** (Biochemistry, Graduated with Honors): Karazin Kharkiv National University, Kharkiv, Ukraine

Affiliations

2001-2002 – BSc Diploma Student, State Scientific Center of Drugs of the Ministry of Health, Academy of Sciences of Ukraine, Kharkiv, Ukraine

2002-2003 – MSc Diploma Student, Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Puschino, Russian Federation

2004–2011 – PhD Student, Institute of Molecular Genetics Academy of Sciences of the Czech Republic, v.v.i., Prague, Czech Republic

Special courses

April 14-21, 2007 – ENII Summer School in Advanced Immunology, Capo Caccia, Sardinia, Italy

May 12-16, 2008 - Acquisition and Processing of the Image in Microscopy, Prague, Czech Republic

Main research interests

Infectious diseases and immune response, various approaches for detection and quantification of pathogens, mapping and functional analysis of genes that control susceptibility and resistance to infections.

Experience with methods

PCR, PCR-ELISA, light and fluorescent microscopy, histological analysis of tissue sections, immunohistochemistry, ELISA, DNA/RNA isolation, reverse transcription, restriction analysis, electrophoresis, preparation of organs from animals, statistical analysis, sequencing, cell cultures, isolation of cells from blood.

Current participation in research projects

1. Mapping of genes that control parasite numbers in *Leishmania major* and *Leishmania tropica* infection.

2. Development of drugs for treatment of leishmaniasis.

3. Histopathological changes after *Leishmania* infection.

4. Immunohistochemical detection of molecules that take part in host-parasite interactions.

Main international conferences

September 6-9, 2006 – 16th European Congress of Immunology, Paris, France

T. Kobets, H. Havelková, I. Kurey, I. Grekov, J. Badalová, M. Svobodová, K. Trtková, M. Lipoldová. Mapping of genes that control *Leishmania* parasite number. Book of abstracts, p. 382.

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August 21-25, 2007 – 13th International Congress of Immunology, Rio de Janeiro, Brazil.

I. Kurey, **T. Kobets**, H. Havelková, M. Slapničková, K. Trtková, I. Grekov, P. Demant, M. Lipoldoavá. Genetic control of parasite dissemination in *Leishmania major* infection – specificity and complex relation to local and systemic disease. On CD.

February 3-7, 2009 – 4th World Congress of Leishmaniasis Lucknow, India.

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Publications

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In preparation...