Third Faculty of Medicine, Charles University in Prague

Institute of Molecular Genetics AS CR, v.v.i., Prague, Czech Republic

Leishmania tropica: immunopathology and genetic control

Thesis for the degree of Philosophiae Doctor (PhD)

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By: Yahya Sohrabi M.Sc.

Supervisor: Doc. Marie Lipoldová, PhD

Institute of Molecular Genetics AS CR, v. v. i.

Consultant: Dr. Ali Khamesipour, PhD

Center for Research & Training in Skin Diseases & Leprosy, Tehran University of Medical Sciences

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Abstract

Leishmaniasis is a neglected tropical disease caused by protozoan parasites of the genus *Leishmania* and transmitted by female sand flies. The outcome of *Leishmania* infection depends both on host and pathogen factors. There are three major clinical forms of leishmaniasis: cutaneous, mucocutaneous and visceral. Similarly as *L. major*, *L. tropica* very often causes cutaneous leishmaniasis in humans, but in rare occasions can also visceralize and cause systemic disease. Leishmaniasis caused by *L. tropica* has become a major public health problem in different endemic foci due to recent outbreaks in several urban areas and spread to new regions. The complications of the disease and lack of safe, effective and affordable drug and vaccine against *L. tropica* infection require considerable attention to studies of the host-*L. tropica* interaction. Until recently, the research of leishmaniasis caused by *L. tropica* was limited due to lack of suitable inbred model and difficulties in inducing infection in animals.

This thesis summarizes the results of my PhD project devoted to development of a suitable mouse model of the infection caused by *L. tropica*, study of mechanisms of the disease, and also mapping controlling genes/loci. We analyzed susceptibility to *L. tropica* infection using recombinant congenic (RC) CcS/Dem strains. We identified CcS-16 and CcS-9 as highly susceptible strains to *L. tropica* infection. We showed that CcS-9 strain not only has large lesions but also developed visceral pathology after infection with *L. tropica*. Based on the results of massive histological analysis and gene expression we showed that probably the intensive inflammation in visceral organs and skin is the main reason for splenomegaly, hepatomegaly and large skin pathology in CcS-9 infected mice. In F_2 hybrids between BALB/c and CcS-16 we detected and mapped eight gene-loci, *Ltr1-8* (*Leishmania tropica* response 1-8) that control various manifestations of disease: skin lesions, splenomegaly, hepatomegaly, parasite numbers in spleen, liver, and inguinal lymph nodes, and serum level of CCL3, CCL5, and CCL7 after *L. tropica* infection. These results represent the first mapping of *L. tropica* susceptibility loci in any species. In our human study we identified the CD8+ T cells as the main producer of IFN-γ which might be the responsible cells for maintenance of protective immune response against human leishmaniasis.

The availability of an informative animal model enabled us to study mechanisms of the disease and map gene/loci participating in the control of resistance to *L. tropica.* This knowledge may also provide hints for the development of novel strategies of the disease control. Further deep characterization of *L. tropica* infection in CcS-9 and CcS-16 strains may help to understand the detailed mechanisms of the disease and would open new perspectives of the research, treatment and vaccine development against leishmaniasis caused by this parasite. The present data about CD8+ T cells might be used as a basis for future investigations of memory T cells in human leishmaniasis and might have an implication in the development of an effective vaccine.

Abstrakt

Leishmanióza je opomíjená tropická nemoc působená prvočím parazitem rodu *Leishmania*, který je přenášen samičkami flebotomů. Rozdílné projevy infekce leishmaniemi závisí na faktorech spojených s hostitelem i patogenem. Existují tři hlavní formy leishmaniózy: kožní, kožně-slizniční a viscerální. Podobně jako *L. major* i *L. tropica* často způsobuje u člověka kožní leishmaniózu, ale ve výjimečných případech může také visceralizovat a vést k systémovému onemocnění. Kvůli propuknutí nemoci v několika městských oblastech a jejímu proniknutí do nových regionů se leishmanióza způsobená parazitem *L. tropica* stala jedním z hlavních problémů veřejného zdraví v těchto různých endemických ohniscích. Komplikace spojené s nemocí a chybějící bezpečný a efektivní lék a vakcína proti infekci *L. tropica* vyžadují, aby byla studiu interakcí hostitele a patogenu věnována zvýšená pozornost.. Donedávna byl výzkum leishmanózy způsobené parazitem *L. tropica* limitován neexistencí vhodného zvířecího inbredního modelu a problémy při vyvolání infekce u zvířat.

Tato práce shrnuje vysledky mého PhD projektu věnovaného vyvinutí vhodného myšího modelu infekce způsobené *L. tropica*, studiu mechanismů nemoci a mapování kontrolních genů/lokusů. Analyzovali jsme vnímavost k infekci *L. tropica* s použitím rekombinantních kongenních (RC) CcS/Dem myších kmenů. Identifikovali jsme kmeny CcS-16 a CcS-9 jako vysoce vnímavé k infekci *L. tropica*. Dále jsme ukázali, že kmen CcS-9 kromě velkých kožních lezí vykazoval i viscerální patologii. Na základě výsledků velké histologické analýzy a genové exprese jsme ukázali, že intenzivní zánět ve viscerálních orgánech a kůži je pravděpodobně hlavním důvodem pro splenomagalii, hepatomegalii a velkou kožní patologii u CcS-9 infikovaných myší.

Pomocí F_2 hybridů mezi BALB/c a CcS-16 jsme detekovali a zmapovali 8 genů-lokusů *Ltr1-8* (*Leishmania tropica* response), které kontrolují různé projevy nemoci: kožní leze, splenomegalii, hepatomegalii, počty parazitů ve slezinách, játrech a inguinálních lymfatických uzlinách a hladinu CCL3, CCL5 a CCL7 v séru po infekci *L. tropica*. Tato práce představuje první úspěšné mapování genů vnímavosti k *L. tropica* u jakéhokoliv živočicha.

Příbuzný projekt s lidskými vzorky popsaný v poslední části práce identifikoval CD8 T buňky jako hlavní producenty IFN-γ. Tyto buňky tedy mohou být odpovědny za udržování protektivní imunitní reakce proti lidské leishmanióze.

Dostupnost informativního zvířecího modelu umožnila studovat mechanismy nemoci a mapování genů/lukusů podílejících se na kontrole rezistence k *L. tropica*. Tyto znalosti mohou naznačit nové strategie kontroly choroby. Další podrobná charakterizace infekce *L. tropica* u kmenů CCS-9 a CCS-16 může pomoci pochopit detailní mechanismy onemocnění a otevřít nové perspektivy výzkumu, léčení a vývoji vakcíny proti leishmanióze způsobené tímto parazitem.

Introduction

Leishmaniasis is a neglected tropical disease caused by protozoan parasites of the genus *Leishmania*. Leishmaniasis is a vector-borne disease transmitted by female sand flies. Parasites reside in neutrophils, monocytes and macrophages, as well as in dendritic cells and fibroblasts (Lipoldová and Demant 2006, Bogdan 2008). Leishmaniasis is endemic in 98 countries on 5 continents, causing 20,000 to 40,000 deaths per year (Alvar 2012). Leishmaniasis is endemic in areas of tropics, subtropics, and southern Europe, in setting ranging from rain forests in the Americas to desert western Asia (Herwaldt 1999). The outcome of *Leishmania* infection depends both on host and pathogen factors. The disease comprises a diverse array of clinical forms, ranging from non-lethal cutaneous leishmaniasis (CL) to potentially lethal visceral leishmaniasis (VL). The control of intercellular bacteria and protozoa, including *Leishmania*, usually requires CD4+ T cells and IFNγ and/or TNF-dependent activation of macrophages (Lipoldová and Demant 2006). 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 inducible nitric oxide synthase (iNOS) alone is not necessarily sufficient to control *Leishmania* (Bogdan 2008). Recovery from CL is usually accompanied with long-lasting protection and induction of strong immune response. The phenotypes, generation and maintenance of central (=T(CM)) and effector (=T(EM)) memory T cell subsets in human leishmaniasis are not well known (Khamesipour 2012). 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). *L. tropica* very often causes cutaneous leishmaniasis in humans, but in rare occasions can also visceralize and cause systemic disease (Sohrabi 2013). Leishmaniasis cause by *L. tropica* has become a major public health problem in different endemic foci due to recent outbreaks in several urban areas and spread to new regions (Ready 2010). The complications of the disease and lack of safe and effective vaccine and affordable drug and against *L. tropica* infection require considerable attention to studies of the host-*L. tropica* interaction. Until recently, the research of leishmaniasis caused by *L. tropica* was limited due to lack of suitable inbred model and difficulties in inducing infection in animals (Anderson 2008).

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). Animal models help to overcome problems of human studies and understand the genetic basis of polygenic diseases. Use of recombinant congenic mouse strains (RCS) made a great contribution to the study of complex diseases (Demant 2003), including leishmaniasis. These strains differ greatly in susceptibility to *L. major* due to random distribution of 12.5% of STS genome on 87.5% BALB/c background in their genomes.

Development of lesions after infection with *L. tropica* in commonly used strains of mice like BALB/c or C57BL/6, rats and hamsters is slow or not apparent (Lira, Mendez et al. 1998; Svobodová,

Votýpka et al. 2003) (Svobodová M 2003; Anderson, Lira et al. 2008). Golden hamster (*Mesocricetus auratus*) was considered to be the best model host of the *L. tropica*, but this host is not inbred. Only several strains of *L. tropica* have been described to cause cutaneous disease in inbred BALB/c mice (Lira, Mendez et al. 1998) (Girginkardesler, Balcioglu et al. 2001), thus providing a better defined host. In contrast to the extensive data about biology of immune response to *L. major* infection (reviewed in (Sacks and Noben-Trauth 2002)) and its genetic control (Lipoldová and Demant 2006)), there is relatively few information about specific immunological mechanisms against *L. tropica* infection. Developing a well-defined model for study of *L. tropica* would contribute to understanding of the clinical course of infection and present different aspects of pathology in human.

Aim of the study

I. Development, defining and characterization of suitable model for studying *L. tropica* infection
II. Studying mechanism of the disease and genetic control of susceptibility to *L. tropica* infection
III. Phenotyping and defining the cells responsible for maintenance of protective immune response in human leishmaniasis

Materials and methods

1) We studied susceptibility to *L. tropica*, using BALB/c-c-STS/A (CcS/Dem) recombinant congenic (RC) strains, which differ greatly in susceptibility to *L. major*. Mice were infected with *L. tropica* and skin lesions, cytokine and chemokine levels in serum, and parasite numbers in organs were measured.

2) We analyzed skin and visceral pathology in CcS-9 strain after infection with *L. tropica*. We estimated parasite number in organs, infiltration of different cells and expression of genes in the tissues.

3) We analyzed genetics of response to *L. tropica* in infected F_2 hybrids between BALB/c×CcS-16. CcS-16 strain carries STS-derived segments on nine chromosomes. We genotyped these segments in the F_2 hybrid mice and tested their linkage with pathological changes and systemic immune responses.

4) We analyzed profile of T cell subsets on peripheral $CD8^+$ T cells from volunteers with history of cutaneous leishmaniasis (HCL). We isolated CD8+ T cells, or CFSE labelled CD8+ T cells or purified CD8+CD45RO−CD56−CD57− naïve and CD8+CD45RO+CD45RA−CD56−CD57− total memory T cells and cultured with 1:10 of autologous monocyte derived macrophage. We phenotyped the T cell populations and measured cytokine production before and after stimulation with soluble *Leishmania* antigen preparation.

Results

1) Our data showed that females of BALB/c and several RC strains developed skin lesions after infection with *L. tropica*. In some strains parasites visceralized and were detected in spleen and liver. Importantly, the strain distribution pattern of symptoms caused by *L. tropica* was different from that observed after *L. major* infection. Moreover, sex differently influenced infection with *L. tropica* and *L.*

major. *L. major*-infected males exhibited either higher or similar skin pathology as females, whereas *L. tropica*-infected females were more susceptible than males. The majority of *L. tropica*-infected strains exhibited increased levels of chemokines CCL2, CCL3 and CCL5. CcS-16 females, which developed the largest lesions, exhibited a unique systemic chemokine reaction, characterized by additional transient early peaks of CCL3 and CCL5, which were not present in CcS-16 males nor in any other strain.

2) Our analysis of susceptibility to *L. tropica* using CcS/Dem series revealed that CcS-9 strain not only developed large lesion but also it was the only CcS/Dem strain which exhibited visceral pathology. To understand the reasons of the unique symptoms of CcS-9 strain, we evaluated recruitment of different types of immune cells to the tissues, and also performed the gene expression analysis. We demonstrated the unique role of inflammatory factors that orchestrated immune responses through modulation of infiltration of different cell types to organs and tissues reflecting the level of susceptibility to *L. tropica*.

3) We used CcS-16 strain to dissect the genetic susceptibility and functionally characterize the gene-loci regulating the immune responses and pathology due to *L. tropica*. The present project describes the first identification of the genetic loci controlling susceptibility to *L. tropica* by mapping 8 *Ltr* (*Leishmania tropica* response) loci. Individual *Ltr* loci affect different subsets of the disease manifestations, exhibit organ specific effects and a separate control of parasite load and organ pathology. We observed multiple gene interactions controlling symptoms during *L. tropica* infection. *Ltr2*, *Ltr3*, *Ltr6* and *Ltr8* showed single gene effect, while *Ltr1*, *Ltr4*, *Ltr5* and *Ltr7* were detected only in gene-gene interactions with other *Ltr* loci. Interestingly, *Ltr3* exhibited the phenomenon of transgenerational parental effect on parasite numbers in spleen. *Ltr1*, which controlls parasite number in lymph nodes, was the most precisely mapped locus (4.07 Mb). Comparative analysis showed that five *Ltr* loci co-localized with previously identified loci controlling susceptibility to *L. major*, whereas three were likely *L. tropica* specific.

4) The related project described in the last part of the thesis, identified the $CD8^+$ T cells as the main producer of IFN-γ which might be the responsible cells for maintenance of protective immune response against human leishmaniasis. Our study showed that in group with history of cutaneous leishmaniasis (HCL) and control groups, mean frequencies of $CCR7^+CD45RA^+CD8^+$ naïve and $CCR7^-CD45RA^-CD8^+$ T(EM) cells were higher than other subsets before culture, but after stimulation with soluble *Leishmania* antigen, the frequency of naïve T cells was significantly decreased and the frequency of T(EM) cells was significantly increased. T (EM) phenotype composed the highest portion of proliferating Carboxy Fluorescein diacetate Succinimidyl Ester (CFSE)-dim population which was significantly higher in HCL volunteers than in control group. Stimulation of isolated $CD8^+$ memory T cells, but not naïve T cells, from HCL volunteers induced a significantly higher IFN-γ production compared with that of healthy controls. Intracellular IFN-γ staining provided the same result. Memory population is shown to be responsible for *Leishmania*-induced IFN-γ production. *Leishmania*-reactive proliferating T(EM) cells were identified as the most frequent subset.

Conclusions

In general the thesis presents the new insight to the mechanisms of leishmaniasis caused by *L. tropica*. These studies provided experimental evidences that the host genotype and the host-*L. tropica* interactions significantly contributed to risk and development of the disease.

We established the first reliable mouse model for genetic studies of *L. tropica* infection. Comparison of *L. tropica* and *L. major* infections indicates that the strain patterns of response are species specific, with different sex effects and largely different host susceptibility genes. All tested strains contained parasites in inguinal lymph nodes. Some strains contained parasites also in spleen and liver, but none of the tested strains developed splenomegaly or hepatomegaly. Females of strain CcS-16, which developed the largest lesions, exhibited a unique systemic chemokine reaction, characterized by early peaks of CCL3 and CCL5 in serum, which were not present in CcS-16 males nor in any other strain.

These results also illustrate the contribution of inflammation and infiltration of various cells to the outcome of *L. tropica* infection. We have found that RC strain CcS-9 differs from parental strains and exhibits large skin lesions, splenomegaly and hepatomegaly. The difference in pathology from the susceptible strain BALB/c is not due to higher parasite numbers, but is caused by more severe inflammatory response, characterized by higher numbers of infiltrating cells and expression of inflammatory molecules.

We have also identified the first identification of genetic loci controlling susceptibility to *L. tropica* in any species. The different combinations of alleles controlling various symptoms of the disease likely co-determine different manifestations of disease induced by the same pathogen in individual mice.

Finally we have shown that memory CD8+ T cells are the main source of IFN-γ in people with history of leishmaniasis. This can be considered to be potential therapeutic target for those nonhealed patients who do not respond to drugs and do not heal.

All together this knowledge could provide hints for the development of novel strategies of the disease control. Further deep characterization of *L. tropica* infection in CcS-9 and CcS-16 strains may help to understand the detailed mechanisms of the disease and would open new perspectives of the research, treatment and vaccine development against leishmaniasis caused by this parasite. Also our results about frequency of $CD8^+$ memory subset in CL may implicate their role in recall immune response and protection against *Leishmania* infection. The present data might be used as a basis for future investigations of memory T cells in human leishmaniasis and might have an implication in the development of an effective vaccine.

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Curriculum vitae

Yahya Sohrabi M.Sc.

Date and Place of Birth: 28 August, 1978, Hamedan, Iran

Education

B.Sc. Microbiology, Department of Basic Science, Azad University, Qom, Iran, 2001
M.Sc. Microbiology, Department of Basic Sciences, Azad University, Qom, Iran 2005

Working experience:

Research fellow and PhD student at the Institute of Molecular Genetics, Academy of Sciences, Czech Republic, 2007-

Research Fellow at Jamia Hmadard University and Jawaharlal Nehru University, New Delhi- Feb - Oct 2006

Research Assistant at the Center for Research and Training in Skin Diseases and Leprosy, 2001- 2006

Lab experience: PCR, Real-time PCR, Microarray data analysis, Electrophoresis, Gel document and SDS-page, ELISA, Antibody and cytokine assay, Lowry and Bradford protein assay , Cell and lymphocyte culture, MACS and FACS, handling and work with animal model of leishmaniasis, parasite burden test, Liposome preparation and etc.

Research interests

Immunology of leishmaniasis, mapping and functional analysis of genes that control susceptibility to leishmaniasis.

Selected abstracts and oral presentation

Sohrabi Y., Slapnickova M., Volkova V., Kobets T., Havelkova H., Vojtiskova J., Svobodova M., Demant P. Lipoldova M., First model of visceral disease after infection with *Leishmania tropica* **Fifth World Congress on Leishmaniasis (WorldLeish5), Porto de Galinhas, Pernambuco, Brazil, May 13th to 17th, 2013**

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