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Genotype influence on development of infections caused by *Trypanosomatidae* in mouse

Vliv genotypu na průběh infekcí působených různými druhy čeledi *Trypanosomatidae* u myši

Ph.D. thesis

Supervisor: Assoc. Prof. Marie Lipoldová, Ph.D.

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Prohlášení

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Abstract

Parasitic protists of genera *Trypanosoma* and *Leishmania* are members of *Trypanosomatidae* family. In our studies, we investigated genetic influence on infections caused by these parasites in a mouse model. These diseases are on genetic level controlled by quantitative trait loci (QTLs), when the resulting phenotype is controlled by set of genes with small individual effect. As a mouse model for mapping of QTLs controlling these infections, we used recombinant congenic strains (RCS). Each RCS carry unique set of 12.5% of the genome from donor parental strain on genetic background of other parental strain. For mapping of QTLs controlling infections caused by *Trypanosoma brucei brucei* (*T. b. brucei*) and *Leishmania tropica* (*L. tropica*) and eosinophil infiltration into inguinal lymph nodes after *Leishmania major* (*L. major*) infection, we used RCS from CcS/Dem series, where STS is donor strain and BALB/cHeA is strain of genetic background. First, it was necessary to find suitable model strains for mapping. In all three studies, we selected RCS, which exceeded range of monitored phenotype parameters in comparison with any other tested RCS or parental strains. Mice of RCS CcS-11 showed shorter survival after *T. b. brucei* infection and strain CcS-9 exhibited higher eosinophil infiltration after *L. major* infection. For analysis of genetic control of susceptibility to *L. tropica*, we selected females of the strain CcS-16, which were previously described to have larger lesions and unique chemokine reaction after *L. tropica* infection. In experiments with F_2 hybrids of these strains and background parental strain BALB/cHeA we were able to map four novel loci controlling *T. b. brucei* infection (*Tbbr1-4*), eight loci controlling *L. tropica* infection (*Ltr1-8*) and four loci controlling eosinophil infiltration after *L. major* infection (*Lmr14*, *Lmr15*, *Lmr25* and *Lmr26*). In the segments covering these loci, we found many genes, which were previously described to have a role in investigated infections or eosinophil function but the observed phenotypes can be also controlled by genes with unknown functions in response to these infections, eosinophil function or in immune system in general. We observed strong sex influence in all three response to infections. The difference in survival after *T. b. brucei* infection was more prominent in CcS-11 females than males,

larger lesions and unique chemokine reaction was observed only in *L. tropica* infected CcS-16 females and loci *Lmr15* and *Lmr26* controlled eosinophil infiltration after *L. major* infection only in male mice. Many of newly discovered loci overlap with each other, with previously described loci controlling infections caused by *Trypanosomatidea* family or loci controlling other infections and therefore can share same controlling mechanisms. Next important step in this research will be mechanistic explanation of influence of the discovered loci/genes on disease phenotypes.

Keywords:

Trypanosomatidae, *Trypanosoma*, *Leishmania*, Eosinophil infiltration, Quantitative trait loci, Mouse model

Abstrakt

Parazitičtí prvoci rodu *Trypanosoma* a *Leishmania* jsou členi čeledi *Trypanosomatidae*. V naší práci jsme na myším modelu zkoumali vliv genotypu na infekce způsobené těmito parazity. Tato onemocnění jsou na genetické úrovni řízena lokusy kvantitativních vlastností (QTL), kdy výsledný fenotyp je řízen množinou genů s malým individuálním účinkem. Jako myší model pro mapování QTL, které kontrolují tyto infekce, jsme použili rekombinantní kongenní kmeny (RCS). Každý RCS nese jedinečnou kombinaci 12,5% genomu rodičovského kmene dárce na genetickém pozadí druhého rodičovského kmene. Pro mapování QTL kontrolujících infekce *Trypanosoma brucei brucei* (*T. b. brucei*) a *Leishmania tropica* (*L. tropica*) a infiltraci eosinofilů do inguinálních uzlin po infekci *Leishmania major* (*L. major*) jsme použili RCS z řady CcS/Dem, kde STS je kmen dárce a BALB/cHeA je kmen genetického pozadí. Nejprve bylo nutné najít pro mapování vhodné modelové kmeny. Ve všech třech studiích jsme zvolili RCS, který překročil rozsah monitorovaných parametrů fenotypu ve srovnání s jinými testovanými RCS i rodičovskými kmeny. Myši RCS CcS-11 vykazovaly kratší přežití po infekci *T. b. brucei* a myši kmene CcS-9 vykazovaly vyšší infiltraci eosinofilů po infekci *L. major*. Pro analýzu genetické kontroly vnímavosti k *L. tropica* jsme vybrali samice kmene CcS-16, u kterých byly po infekci *L. tropica* popsány větší léze a jedinečná chemokinová reakce. Experimenty s F_2 hybridy mezi těmito kmeny a rodičovským kmenem BALB/cHeA umožnily zmapovat čtyři nové lokusy kontrolující infekci způsobenou *T. b. brucei* (*Tbbr1-4*), osm lokusů kontrolujících infekci způsobenou *L. tropica* (*Ltr1-8*) a čtyři lokusy kontrolující infiltraci eosinofilů po infekci *L. major* (*Lmr14*, *Lmr15*, *Lmr25* a *Lmr26*). V segmentech pokrývajících tyto lokusy jsme nalezli mnoho genů, u kterých byla dříve popsána role u zkoumaných infekcích nebo u funkce eozinofilů. Pozorované fenotypy mohou být ale také kontrolovány geny, u kterých není známa spojitost s těmito infekcemi, eozinofilní funkcí nebo imunitním systémem obecně. U všech tří odpovědí na infekce jsme pozorovali silný vliv pohlaví. Rozdíl v přežití po infekci *T. b. brucei* byl výraznější u samic CcS-11 než u samců, větší léze a jedinečná chemokinová reakce byly pozorovány pouze u samic CcS-16

infikovaných *L. tropica* a *Lmr15* a *Lmr26* kontrolují infiltraci eosinofilů po infekci *L. major* pouze u samců. Řada nově objevených lokusů se překrývá navzájem, s dříve popsanými lokusy kontrolujícími infekce způsobené čeledí *Trypanosomatidea* nebo s lokusy kontrolujícími jiné infekce a mohou tak sdílet stejné kontrolní mechanismy. Dalším důležitým krokem v tomto výzkumu bude funkční vysvětlení vlivu objevených lokusů/genů na fenotypy onemocnění.

Klíčová slova:

Trypanosomatidae, *Trypanosoma*, *Leishmania*, Infiltrace eosinofilů, Lokusy kvantitativních vlastností, Myší model

Contents

List of abbreviations

AAT	Animal African Trypanosomiasis
Adcy3	Adenylate cyclase 3
AIL	Advanced intercross line
APC	Antigen presenting cell
Bcl2l1	B-cell lymphoma 2-like 1
bg/Lyst	Beige/Lysosomal trafficking regulator
CCL1	Chemokine (C-C motif) ligand 1
CCL11	Chemokine (C-C motif) ligand 11
CCL2	Chemokine (C-C motif) ligand 2
CCL3	Chemokine (C-C motif) ligand 3
CCL4	Chemokine (C-C motif) ligand 4
CCL5	Chemokine (C-C motif) ligand 5
CCL7	Chemokine (C-C motif) ligand 7
CCL8	Chemokine (C-C motif) ligand 8
CCRL2	Chemokine (C-C motif) receptor-like 2
Cd2	CD2 antigen
Cd40	CD40 antigen
Cd44	CD44 antigen
Cd74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)
Chr	Chromosome
Cish	Cytokine inducible SH2-containing protein
CL	Cutaneous leishmaniasis
cM	Centimorgan
CNS	Central nervous system
CSS	Chromosome substitution strains
CXCR1	Chemokine (C-X-C motif) receptor 1
CXCR2	Chemokine (C-X-C motif) receptor 2
DC	Dendritic cell
Dice1b	Determination of interleukin 4 commitment 1b
Dll4	Delta-like 4 (Drosophila)
Dnmt3a	DNA methyltransferase3a
FCN2	Ficolin-2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Gnai2	Guanine nucleotide binding protein (G protein), alpha inhibiting 2
GTM	Genome-tagged mice
H2	histocompatibility-2, MHC
HAT	Human African Trypanosomiasis
Hck	Hemopoietic cell kinase
Hdc	Histidine decarboxylase
HIV	Human imunodeficiency virus
HLA	Human leukocyte antigen
Hmgb1	High mobility group box 1
Ifnb1	Interferon beta 1, fibroblast
IFNGR1	Interferon gamma receptor 1
IFNγ	Interferon gamma
IgE	Imunoglobulin E
Igf1	Insulin-like growth factor 1
IL1	Interleukin 1
Il1	Interleukin 1 complex
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-4	Interleukin 4
Il12a	Interleukin 12a

Il13	Interleukin 13
Il3	Interleukin 3
Il4	Interleukin 4
Il5	Interleukin 5
IL-6	Interleukin 6
Ir2	Immune response-2
Ity7	immunity to *S. typhimurium* 7
Jun	Jun oncogene
L. amazonensis	*Leishmania amazonensis*
L. braziliensis	*Leishmania braziliensis*
L. donovani	*Leishmania donovani*
L. infantum	*Leishmania infantum*
L. major	*Leishmania major*
L. mexicana	*Leishmania mexicana*
L. tropica	*Leishmania tropica*
Lgals3	Lectin, galactose binding, soluble 3
Lmr	Leishmania major response
Ltr	*Leishmania tropica* response
Man2a1	Mannosidase 2, alpha 1
Mbd2	Methyl-CpG binding domain protein 2
MBL2	Mannose-binding lectin (protein C) 2
MCP-1	Monocyte chemotactic protein-1
MCP-3	Monocyte chemotactic protein-3
MHC	Major histocompatibility complex
Mif	Macrophage migration inhibitory factor
MIP-1α	Macrophage inflammatory protein-1α
MIP-1β	Macrophage inflammatory protein 1-β
ML	Mucocutaneous leishmaniasis
Mmp9	Matrix metallopeptidase 9
MΦ	Macrophage
Ncoa1	Nuclear receptor coactivator 1
NECT	Nifurtimox–eflornithine combination therapy
Ngf	Nerve growth factor
Nlrp3	NOD-like receptor family, pyrin domain-containing protein 3
Nos2	Nitric oxide synthase 2, inducible
Notch2	Notch 2
Nramp1/Slc11a1	Natural resistance-associated macrophage protein 1/Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1
p.i.	Post infection
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
Pomc	Pro-opiomelanocortin-alpha
Pram1	PML-RAR alpharegulated adaptor molecule 1
Ptger3	Prostaglandin E receptor 3 (subtypeEP3)
Ptgfr	Prostaglandin F receptor
Ptpn1	Protein tyrosine phosphatase, non-receptor type 1
QTL	Quantitative trait locus
RANTES	Regulated upon activation, normal T-cell expressed, and secreted
RCS	Recombinant congenic strains
RIS	Recombinant inbred strains
Sec22b	SEC22 vesicle trafficking protein homolog B (S. cerevisiae)

Slc7a2	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 2
Smad7	SMAD family member 7
SNP	Single nucleotide polymorphism
SRA	Serum resistance-associated
Stat6	Signal transducer and activator of transcription 6
STR	Short tandem repeat
T. b.	*Trypanosoma brucei*
T. congolense	*Trypanosoma congolense*
T. cruzi	*Trypanosoma cruzi*
Tbbr	*Trypanosoma brucei brucei* response
TGFB1	Transforming growth factor, beta 1
TGFβ	Transforming growth factor beta
Th1	Type 1 T helper cells
Th2	Type 2 T helper cells
Tir	Trypanosome infection response
TNFA	Tumor necrosis factor alpha
TNFB	Tumor necrosis factor beta
TNF-α	Tumor necrosis factor α
Traf6	TNF receptor-associated factor 6
UTR	Untranslated region
Vcam1	Vascular cell adhesion molecule 1
VL	Visceral leishmaniosis
VSG	Variant surface glycoprotein
Vtcn1	V-set domain containing T cell activation inhibitor 1
WHO	World Health Organization

1 Introduction

1.1 Mapping quantitative trait loci controlling infections using mouse model

Complex infectious diseases, such as trypanosomiasis and leishmaniasis, are on genetic level controlled by quantitative loci. It means that responses to these diseases are influenced by many genes with small individual effects on observed phenotypes. Genetic loci which control such complex phenotypes are called quantitative trait loci (QTLs) [1]. The mapping of QTLs in human population may be complicated due to the heterogeneity of outbred human population, variability in allelic frequencies, gene-gene interactions, the incomplete penetrance of disease-causing alleles, and environmental factors such as nutrition, medical care, and hygienic conditions [2]. These problems may be overcome by using of inbred mouse strains in standardized laboratory conditions as a disease model. Mouse and human genomes are highly homologous [3] and mouse is a powerful model for studying of human diseases [4]. For mapping of QTLs in mouse model was developed many mouse strains with different genetic arrangement. These mice were prepared by specific breeding strategies. Basic principle lies in dissection of QTLs/genes that are responsible for different phenotypes in different mouse strains. This can be achieved by experiments on suitable mouse strains where for localizing of controlling loci are used genetic markers [5].

One possibility is using recombinant inbred strains (RIS). RIS are prepared by inbreeding of F_2 hybrids between two parental strains. Each RIS has an unique combination of approximately 50% of the genome from one parental strain and 50 % from other. Usually it is necessary to test more than 20 RIS to map QTLs. Problematic is mapping of QTLs with weak effect on observed phenotype [5, 6].

In our studies we are using recombinant congenic strains (RCS). These inbred strains are genetic combination of two parental strains. Each RCS carry unique combination of approximately 12.5% of the genome from donor parental strain on genetic background of second parental strain. These strains are described in more details in the next chapter.

Another used genetic arrangement are chromosome substitution strains (CSS) also known as consomic strains. Each of these strains has whole one chromosome from one parental strain (donor) on the genetic background of other. To cover whole genome it is needed as many strains as number of chromosomes which is 20 in case of mouse. CSS allow quick finding on which chromosome is given QTL, but if there are more QTLs on one chromosome the result is less informative [5, 7, 8].

Similar to CSS are genome-tagged mice (GTM). These strains do not carry whole chromosome from the donor strain but only a part with an average length of the fragment 23 cM. Each chromosome is covered by multiple strains and for covering of whole mouse genome is necessary approximately 60 strains. GTM have better resolution than CSS [5, 9].

Advanced intercross lines (AILs) allow to detect and map QTLs simultaneously and to shorten chromosomal segments but linkage detection is more difficult and it is necessary to use large number of markers. They represent advanced generation (~F_5-F_8) of crossing of two inbred strains. In each generation are selected mice with greatest heterogeneity for next cross [5, 10]. These strains were also used for mapping of QTLs controlling trypanosomiasis [11] (described in more details in chapter 1.2.1).

Heterogeneous crosses have very different genetic arrangement in comparison with already described models. Instead of crossing of two parental strains, heterogeneous crosses are prepared from several inbred strains. These mice are also prepared with emphasis to be as heterogeneous as possible. At each locus, they can carry one of several possible alleles and more possible QTLs can be detected. For the precise mapping it is necessary to use sufficient number of mice (several thousands) and genetic markers and then it is a powerful tool for finding of QTLs. However, more than two allelic forms in each locus decrease the possibility of their detection and also different interactions for different alleles is more difficult to dissect. It is also necessary to use advanced statistical methods [5, 12, 13].

1.1.1 Recombinant congenic strains

Recombinant congenic strains (RCS) were bred from two parental strains by two backcrosses (N3) followed by inbredisation (intermating subsequent brothers and sisters for about 20 generations). A series of RCS consists of 20 strains and each strain contains approximately 12.5% of genome from the donor parental strain and 87.5% from the background parental strain. Several series of RCS were prepared by combining two different parental strains. These include CcS/Dem series, where the donor strain is STS/A and BALB/cHeA is strain of genetic background (Figure 1), HcB/Dem series (strain C57BL/10ScSnA as donor and C3H/DiSnA as background), OcB/Dem series (strain B10.O20 as donor and O20 as background) [14, 15], and AcB/BcA series (parental strains are A/J and C57BL/6J, donor is either A/J or C57BL/6J on genetic background of other strain) [16].

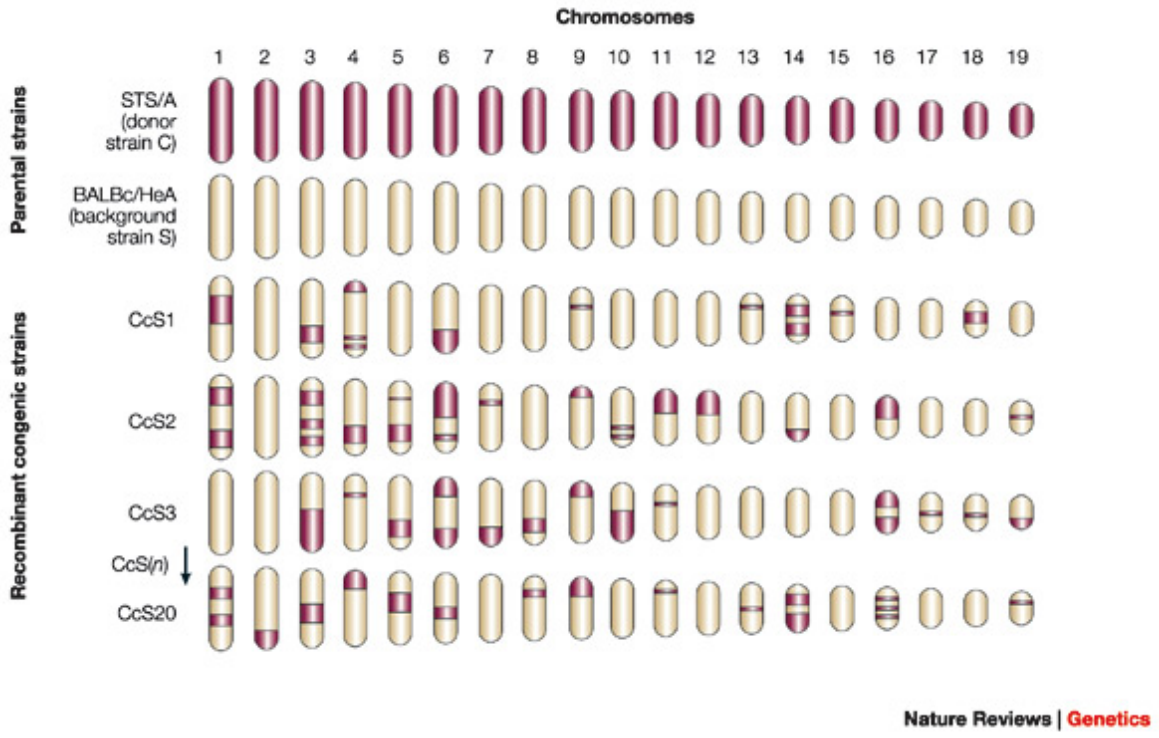

Figure 1. CcS/Dem series of recombinant congenic strains. Figure was adopted from Demant, 2003 [5].

RCS are homozygous at virtually all loci, genetically well characterized and can be screened for desired phenotypes. For QTLs mapping by RCS it is necessary to cross strain back to the background strain and then cross offspring to prepare F_2 generation to prevent distortion caused by possible mutations. With the available genetic markers, it is possible to determine genotype of tested individuals and then map loci/genes controlling given phenotype by linkage analysis. Short tandem repeats (STRs) markers, which use length polymorphism of these repeats in different strains, are typed using PCR and electrophoresis [17, 18]; with single nucleotide polymorphisms (SNPs) markers it is necessary to include cleaving of PCR product by specific restriction enzyme which cleaves only one sequence variant (PCR-RFLP) [19]. The big advantage of RCS is that it is needed just 16-20 markers for linkage analysis, it is possible to map even low-penetrance QTLs with a relatively small amount of mice (200-300) and also interactions between QTLs are relatively easy detectable. The disadvantage is mapping only in a limited part of the genome and therefore many interactions remain undetected [5].

RCS were, for example, used to study genetic control of various types of cancer [5, 20], cytokine-induced activation [21, 22], T-cell proliferative response [23], allogenic reactions [24, 25], cytokine production [26, 27], T cell receptor induced activation [28, 29], *Bordetella pertussis* infection [30], *Salmonella* infection [31, 32], allergic asthma [33], airway hyperresponsiveness [34], and skeletal fragility [35]. RCS system was also introduced into the study of leishmaniasis and it led to better understanding how is this disease genetically controlled and to discovery of many new QTLs controlling this infection in mouse [36]. Genetic control of leishmaniasis is described in more details in chapter 1.2.2.

1.2 *Trypanosomatidae*

Trypanosomatidae family (*Euglenozoa*: *Kinetoplastea*) is a group of diverse parasitic protists. These parasites have two general life strategies: monoxenous species are restricted

only to invertebrate host, while dixenous species shuttle between invertebrates (mainly insect and leeches) and vertebrates (including humans) or plants. To the dixenous group belongs organisms of genera *Trypanosoma*, *Leishmania*, and *Phytomonas* [37]. This thesis deals with clinically important parasites of genera *Leishmania* and *Trypanosoma*.

1.2.1 *Trypanosoma brucei*

According WHO (http://www.who.int/mediacentre/factsheets/fs259/en/) the estimated number of cases of human african trypanosomiasis (HAT, sleeping sickness) caused by *Trypanosoma brucei* (*T. brucei*) is below 15 000 per year. This relatively small number (in 1998 estimated number was 300 000) was achieved by continued control efforts but still this disease is threat for 65 million people in 36 countries of sub-Saharan Africa (Figure 2). Among other factors that need to be improved for better control of the disease such as social, economic and political problems, important issues are using of very old drugs with severe side effects, problematic diagnosis and missing vaccine [38, 39]. For solving of these problems it is necessary to know more about interactions between trypanosome and its host.

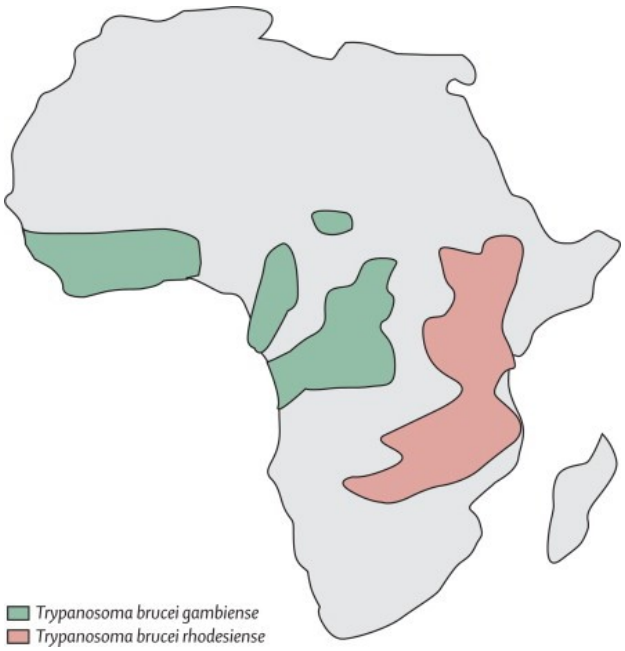

Figure 2. Diagrammatic representation of the distribution of the two types of human African trypanosomiasis in Africa. *Trypanosoma brucei gambiense* (*T. b. gambiense*) causes chronic disease, *Trypanosoma brucei rhodesiense* (*T. b. rhdesiense*) causes acute disease. Figure was adopted from Kennedy, 2013 [38].

T. brucei is unicellular protozoa parasite. During life cycle (Figure 3) alternates between vector – tsetse fly (*Glossina ssp.*) and mammalian host. Upon the bite of the mammalian host by trypanosome-infected tsetse fly the trypanosomes multiply locally in the skin and elicit a local host response in the form of an indurated skin lesion called the chancre. Eventually, the parasites can enter blood stream via lymph vessels and multiply in blood. Tsetse fly can be infected by sucking of blood with trypanosomes [40-42].

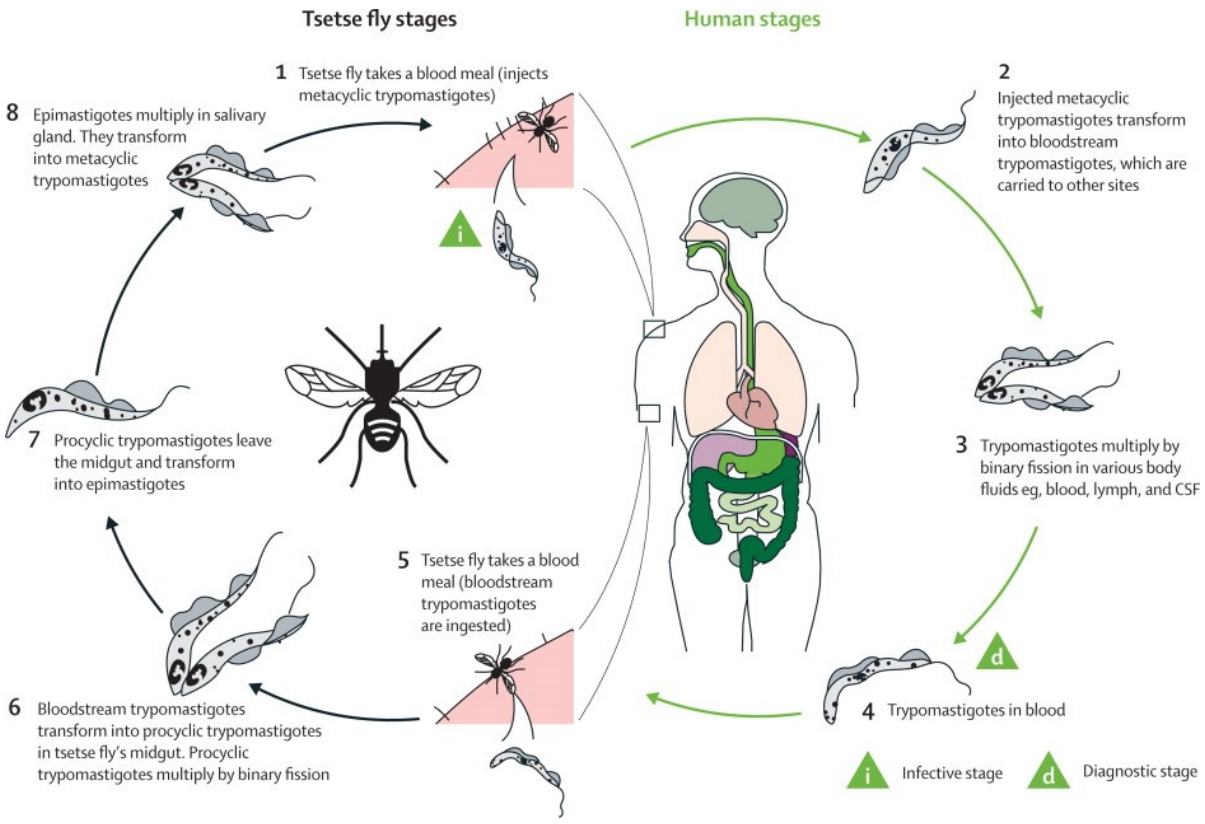

Figure 3. Life cycle of trypanosomiasis. Figure was adopted from Kennedy, 2013 [38].

Trypanosomes have ability to penetrate blood-brain barrier and cause severe brain pathology [43]. Clinical phase of the disease prior to crossing of blood-brain barrier is called early phase and is connected with non-specific symptoms such as fever, headache, malaise and weakness; as the disease progress also specific organs dysfunction can occur (e.g. hearth failure, skin lesions, endocrine disturbances). The late phase starts after

penetrating of blood-brain barrier and getting of trypanosomes into central nervous system (CNS). The late phase is connected with psychiatric disturbances, sleep disorders, motor system disorders, sensory syndromes, abnormal reflexes and if untreated, usually lead to death [44]. The duration of early phase is dependent on subspecies of *T. brucei*. More than 98% of reported cases are caused by *T. brucei gambiense* (*T. b. gambiense*), which is responsible for a chronic disease where the early phase lasts for months or even years after the start of the infection. Acute disease is caused by second subspecies *T. brucei rhodesiense* (*T. b. rhodesiense*) and in these cases the late stage can occur within weeks. Both subspecies also infect animals and domestic animals are important reservoir of the parasite (http://www.who.int/mediacentre/factsheets/fs259/en/). The third major subspecies of *T. brucei* is *T. brucei brucei* (*T. b. brucei*). It is not infective to humans because it lacks the SRA (serum resistance-associated) protein that prevents lysis induced by Apolipoprotein L1, which is present in normal human serum [45, 46]. *T. b. bruce*i is one of the causative agents of animal african trypanosomiasis (AAT, also called Nagana). There are several compounds used for treatment of domestic animals infected by *T. b. brucei* (e.g. suramin, melarsomine). Besides severe side effects, the major problem is resistance of trypanosomes [47].

Trypanosomes use many sophisticated mechanisms to escape destruction by immune system of the mammalian host. One of the most important is an ability of changing a surface coat. On its surface trypanosome has variant surface glycoproteins (VSGs) which form dense coat. Given clone of trypanosome express only one variant of VSG. However, during cell division trypanosome can switch the expressed variant of VSG by replacing the VSG gene in active site with a VSG gene from a silenced locus. Antibody response eliminates first clone, but second clone starts to proliferate and eventually is also eliminated, but new clone appears etc. These cycles are typical for trypanosome infection and continue until exhausting of the immune system (Figure 4). Trypanosome has up to 2000 of VSG genes [48-50]. Trypanosome also causes alteration of basically every component of the immune system. Over-activation of complement leads to hypocomplementemia, anemia and

thrombocytopenia [51]. Soluble variant of VSGs inhibits macrophage production of nitric oxide [52], VSGs cause polyclonal B-cells activation [53]. Trypanosomes also cause suppression of T-cells [54].

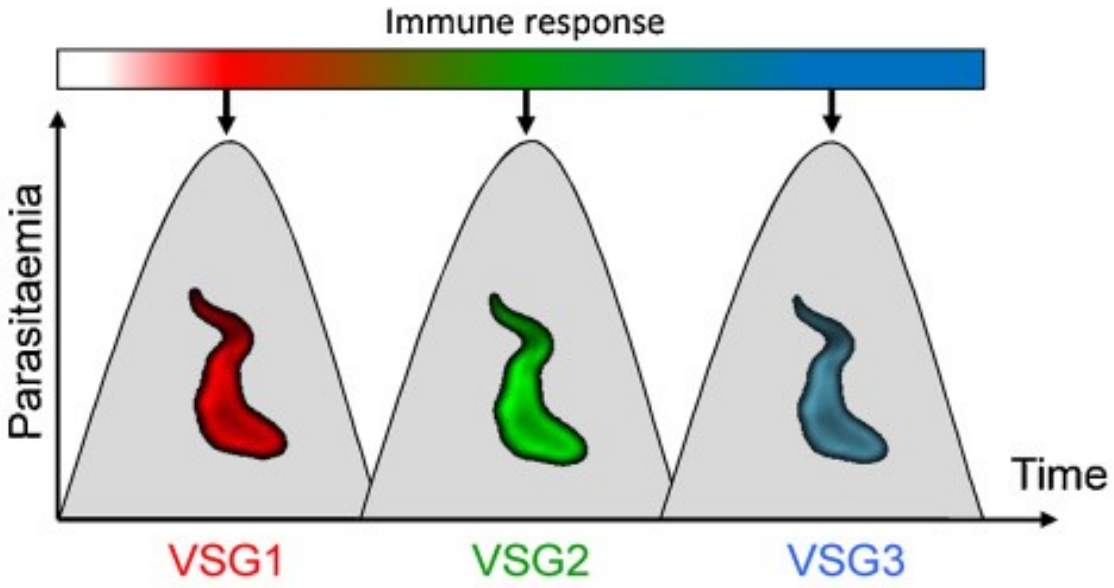

Figure 4. VSGs switching and relapsing parasitaemia. Figure was adopted from Horn, 2014 [50].

For studying of HAT can be used animal infection by *T. b. brucei* which is considered as a suitable model because *T. b. brucei* is closely related to other *T. brucei* subspecies. Many aspects of human disease were discovered in mice model [55]. Different mouse strains react differently to *T. brucei* infection and have different course of the disease. Mouse strains DBA/2, BALB/c, BALB.B, and C3H/He are susceptible with higher parasitemia and shorter survival in comparison with strains C57BL/10, C57BL/6, and C57BL/10(H-2^d) (B10.D2) which are relatively resistant [56, 57]. In other experiment BALB/c mice had higher parasitemia than C57BL/6 mice but these two strains did not differ in survival [58]. Comparison between strains C57BL/6 and 129/SvEv revealed that although 129/SvEv mice have higher parasitemia and lower level of specific IgM (but not IgG), C57BL/6 mice had higher mortality, weight loss and more parasites in CNS [59]. In

study with strains CBA/N, CBA/CaT6 and A/J was observed that strain CBA/N had longer survival, lower level of thymus dependent antibodies and slightly lower splenomegaly but similar parasitemia in comparison with CBA/CaT6 and A/J strains [60]. Although these experiments are not completely comparable because of different experimental condition we can conclude that genetic heterogeneity has great influence on trypanosome infection (Table1).

Table 1. Immune response to *T. brucei* in mouse strains.

Strain	Phenotypes determining response	Response to *Trypanosoma*	Reference
DBA/2	survival, parasitemia	susceptible	[57]
BALB/c	survival, parasitemia	susceptible	[57, 58]
BALB.B	survival, parasitemia	susceptible	[57]
C3H/He	survival, parasitemia parasite differenciation, antibody titre	susceptible	[56, 58]
C57BL/10	survival, parasitemia	resistant	[57]
C57BL/6	survival, parasitemia parasites in CNS. parasite differenciation, antibody titre, weight loss	resistant	[56-59]
B10.D2	survival, parasitemia	resistant	[57]
129/SvEv	survival, parasitemia, parasites in CNS, weight loss, antibody titres	resistant	[59]
CBA/N	survival, antibody titre	resistant	[60]
CBA/CaT6	survival, antibody titre	susceptible	[60]
A/J	survival, antibody titre	susceptible	[60]

Study of *HLA-G* gene, which has an important role in immune system, revealed significant influence of polymorphisms in this gene on HAT susceptibility in human [61].

Three loci controlling susceptibility to trypanosomiasis caused by subgenus *T.* (*Nannomonas*) *congolense* were mapped in mice. *T. congolense* is close relative of *T. brucei* with the same vector and a very similar host-parasite interaction [62]. Controlling loci were named *Tir1* (on chr. 17), *Tir2* (on chr. 5) and *Tir3* (on chr. 1) (for trypanosome infection response). For mapping were used crosses between susceptible strains (BALB/c,

A/J, C3H/HeJ) and resistant strain (C57BL/6) [63]. More detailed mapping divided *Tir3* in three sub-loci named *Tir3a*, *Tir3b* and *Tir3c* [11, 64]. *Tir1* and *Tir2* also control infection by subgenus *T.* (*Schizotrypanum*) *cruzi* (the causative agent of Chagas disease), for mapping was used cross between strains C57BL/6 and B6D2F1, where strain B6D2F1 is F_1 generation of C57BL/6 x DBA/2 cross [65]. Next generation sequencing and other genomic approaches suggested two potential candidate genes at the sites of *Tir* loci: *Pram1* 1 (PML-RAR alpharegulated adaptor molecule 1) at *Tir1* and *Cd244* (natural killer cell receptor 2B4) at *Tir3c* [66].

Loci controlling susceptibility to *T. brucei* were not previously mapped.

1.2.2 *Leishmania*

Leishmaniasis is disease caused by protozoan parasites of genus *Leishmania*. Leishmaniasis occurs in 98 countries on 5 continents (Figure 5) where threats about 350 millions people. It is estimated that leishmaniasis causes 20,000 to 30,000 deaths per year but the actual number may be different due to a lack of relevant data [67], (http://www.who.int/mediacentre/factsheets/fs375/en/). Increasing number of reported cases during last decades is partly due to improved diagnostics, but other factors also play an important role, including drug resistance, lack of effective vector and reservoir control and spreading of *Leishmania* to new areas. Reasons why can Leishmaniasis spread into new areas are mainly urbanization, deforestation and climate changes. The number of cases also influence exposure of susceptible human populations to leishmaniasis as a result of natural disasters, armed conflicts, economic crises and tourism to endemic areas [68]. Prevention and treatment of leishmaniasis is very limited because there is no effective and safe vaccine, and chemotherapy has severe side effects or it's price is too high for wide use in developing countries [69, 70]. A major problem is also coinfection with other infections, e. g. HIV infection [71].

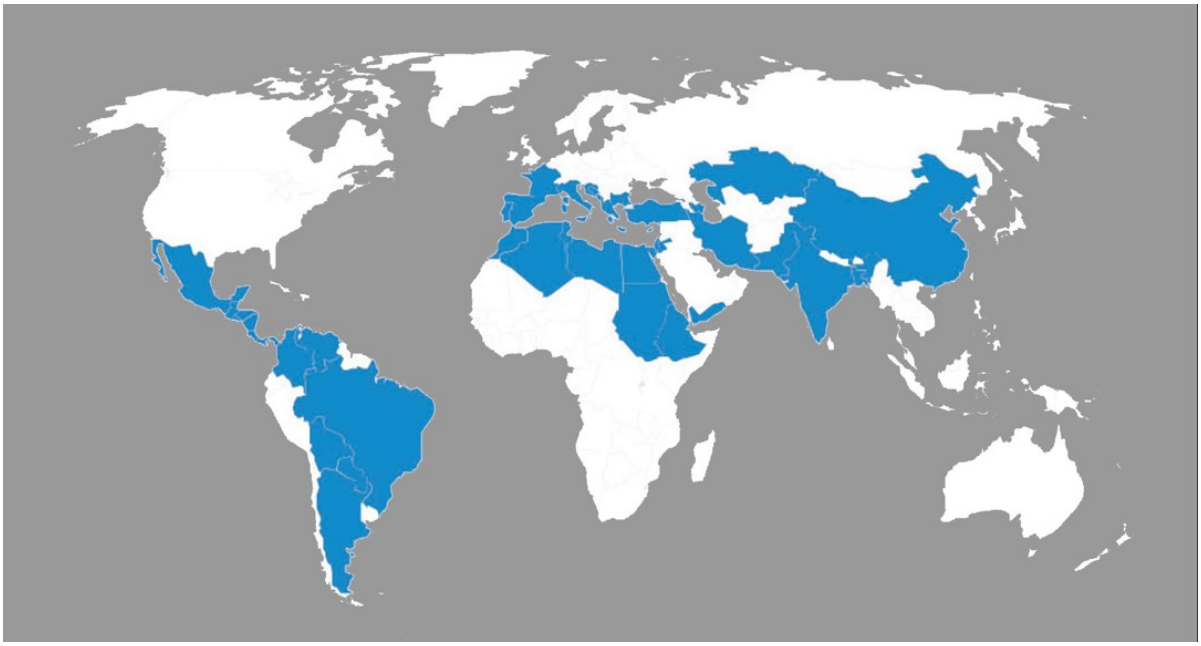

Figure 5. Geographic distribution map of leishmaniasis. Figure was adopted from http://www.bvgh.org.

Leishmania is an intracellular parasite. There are 30 *Leishmania* species of which 21 cause human disease [72], the most important are listed in Table 2. Leishmaniasis is transmitted to vertebrates by *phlebotomine* sandflies. Exceptional cases of transmission by sexual intercourse, blood transfusion and from mother to child were also described [73]. Humans are usually infected by transmission from reservoir animals which are mostly rodents and canids; only some species (*Leishmania tropica* (*L. tropica*) in urban areas is an example) spread from human to human [36].

Table 2. Species of *Leishmania* that cause the disease in human. Table was adopted from Reithinger et al., 2007 [68].

	Main clinical pathology	Transmission cycle	Main geographical distribution
New World *Leishmania* spp			
L (Viannia) braziliensis	LCL, mucosal	Zoonotic	South America, parts of Central America, Mexico
L (Viannia) panamensis	LCL, mucosal	Zoonotic	Northern South America and southern Central America
L (Viannia) peruviana	LCL	Zoonotic	Peru
L (Viannia) guyanensis	LCL	Zoonotic	South America
L (Viannia) lainsoni	LCL	Zoonotic	South America
L (Viannia) colombiensis	LCL	Zoonotic	Northern South America
L (Leishmania) amazonensis	LCL, DCL	Zoonotic	South America
L (Leishmania) mexicana	LCL, DCL	Zoonotic	Central America, Mexico, USA
L (Leishmania) pifanoi	LCL	Zoonotic	South America
L (Leishmania) venezuelensis	LCL	Zoonotic	Northern South America
L (Leishmania) garnhami	LCL	Zoonotic	South America
Old World *Leishmania* spp			
L (Leishmania) aethiopica	LCL, DCL	Zoonotic	Ethiopia, Kenya
L (Leishmania) killicki	LCL	Zoonotic	North Africa
L (Leishmania) major	LCL	Zoonotic	Central Asia, north Africa, middle east, East Africa
L (Leishmania) tropica	LCL	Anthroponotic	Central Asia, middle east, parts of north Africa, southeast Asia
L (Leishmania) donovani	Visceral, LCL	Anthroponotic	Africa, central Asia, southeast Asia
Old and New World *Leishmania* spp			
L (Leishmania) infantum	Visceral, LCL	Zoonotic	Europe, north Africa, Central America, South America

LCL=localised cutaneous leishmaniasis. DCL=diffuse cutaneous leishmaniasis. *Subgenus is given in parentheses. Southeast Asia includes the Indian subcontinent and China

During the life cycle (Figure 6) is *Leishmania* present in several morphological forms. In the infected female *phlebotomine* sandfly parasite exists in a form of extracellular, motile, and long cell with flagellum which is called promastigote. In sandfly gut promastigotes transform to small, rapid swimming metacyclics with elongated flagellum. Metacyclics can be injected to mammalian host during blood-meal by two main

mechanisms: either they can move to the proboscis and be deposited to the skin or reside behind stromodeal valve and can be regurgitated with a backflow of ingested blood which is induced by damage caused by parasite. After inoculation in the host skin antigen presenting cells (APCs) of the host, mainly macrophages (MΦ) and dendritic cells (DC) but also neutrophils and fibroblasts phagocyte metacyclics. In APCs *Leishmania* parasites transform into amastigotes (non-motile, roundish cells without flagellum) which can survive hostile environment of phagosome by inhibiting of leishmanicidal functions, multiply and infect another sandfly during blood-meal. Eventually, they cause breakage of infected cell and enter other cells [36, 74-77].

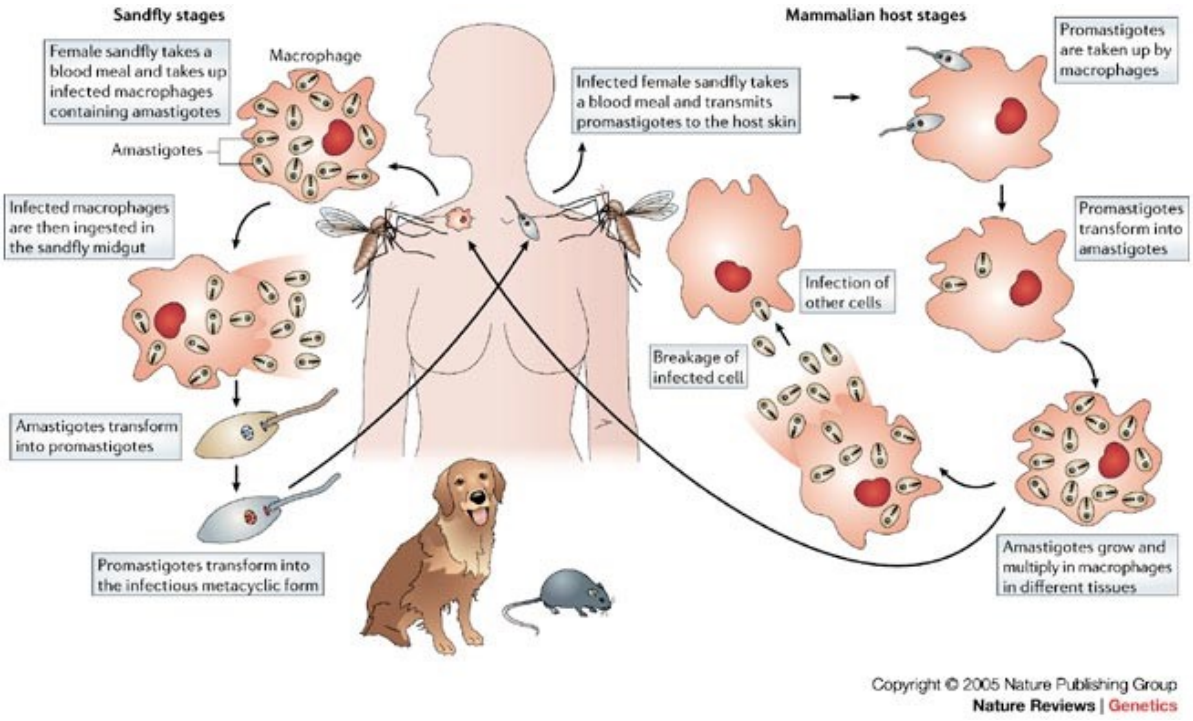

Figure 6. Life cycle of leishmaniasis. Figure was adopted from Lipoldova and Demant, 2006 [36].

Leishmaniasis can be asyptomatic or can cause three main clinical syndromes: cutaneous (CL), mucocutaneous (ML) and visceral leishmaniasis (VL). Cutaneous form can be localized or diffuse and damage is caused by parasites in the dermis. In mucosa parasites cause mucocutaneous form. Parasites can also spread to internal organs such as lymph nodes, liver, spleen, bone marrow, lungs and in rare cases they can get even to brain and

there cause their damage; this form is called visceral leishmaniasis (also known as black fever or "kala-azar"). In organs parasites usually induce inflammatory reaction which leads to the recruitment of inflammatory cells, such as neutrophils, macrophages, eosinophils, and dendritic cells to the site of inflammation. These cells can destroy the parasites by direct phagocytosis and produce cytokines and chemokines, which activate innate and adaptive immune responses. These responses can be either effective and eliminate the parasites or ineffective and lead to chronic inflammation. Developing of a certain form of leishmaniasis in a given individual depends primarily upon parasite species, but it is also determined by the inoculation size, health condition of the host and by the host genetic background [36, 78-82]. Considerable impact also have environmental and social factors [36, 68].

Genotyping of the genes chosen on the basis of previous immunological knowledge detected in human patients influence of polymorphisms in HLA-Cw7, HLA-DQw3, HLA-DR, TNFA (tumor necrosis factor alpha), TNFB, IL4, IFNGR1 (interferon gamma receptor 1) [36], TGFB1 (transforming growth factor, beta 1) [83], IL1 [84], IL6 [85], CCL2/MCP1 (chemokine (C-C motif) ligand 2) [86], CXCR1 (chemokine (C-X-C motif) receptor 1) [87], CXCR2 (chemokine (C-X-C motif) receptor 2) [88], FCN2 (ficolin-2) [89] and MBL2 (mannose-binding lectin (protein C) 2) [90] on response to leishmaniasis. Interestingly several mouse orthologous genes are localized in loci controlling leishmaniasis [91, 92].

Genome-wide linkage analysis in mouse led to the discovery of two susceptibility genes: *Nramp1* (Natural resistance-associated macrophage protein 1)/*Slc11a1* (solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1) [93] and *Fli1* (Friend leukaemia virus integration 1) [94]. These two genes also have impact on human leishmaniasis [87, 95, 96]. *NRAMP1*, which controls susceptibility to *Leishmania donovani* (*L. donovani*) and *Leishmania infantum* (*L. infantum*), is a divalent metal pH-dependent efflux pump at the phagosomal membrane of macrophages, neutrophils and dendritic cells [97]. *NRAMP1* influences antigen presentation by modifying MHC class II expression [98]. Susceptible mouse strains carry a "null" mutation that abolishes gene function (natural

knockout) [99]. At the site of human homolog gene *SLC11A1* was found significant influence of polymorphisms in the promoter, 3'UTR and extragenic region [95]. Gene *Fli1* is linked to wound healing and influences CL caused by *L. major* in mouse [94] and by *L. braziliensis* in human [96].

Natural polymorphisms in genes *bg* (beige)/*Lyst* (lysosomal trafficking regulator) [100] and cationic amino acid transporter *Slc7a2* (solute carrier family 7 (cationic amino acid transporter, y+ system), member 2) [101] influence response to *L. donovani* [102] and *L. major* [101], respectively, but it is not known whether they also play role in human susceptibility.

Strains from CcS/Dem series are combination of highly resistant strain STS (donor) and highly susceptible strain BALB/cHeA (background) and differ greatly in susceptibility to *L. major* [103, 104]. Mainly by using of RCS (CcS/Dem) but also by other mapping techniques were mapped many loci and genes controlling leishmaniasis, these genetic elements are listed in Table 3.

Table 3. Loci and genes identified in leishmaniasis in mice. In table you can find locus/gene name, chromosome localization, *Leishmania* species, controlled trait (in case of cytokines and IgE it is control of their serum levels - unless otherwise indicated, spontaneous proliferation means *in vitro* proliferation of splenocytes from infected mice) and reference. This table is updated version of table from Lipoldova and Demant, 2006 [36].

Locus/gene	Chr	*Leishmania* species	Controlled trait	Reference
Nramp1/ Slc11a1	1	*L. donovani*	Parasite load in liver and spleen	[105, 106]
		L. infantum	Parasite load in liver	[107]
Lmr8	1	*L. major*	IgE	[108]
Lmr20	1	*L. major*	Parasite load in lymph nodes, IgE, IFN-γ	[92]
Ir2	2	*L.donovani*	Parasite numbers, lymphocellular infiltration in liver	[109]
Lmr14	2	*L. major*	Hepatomegaly, splenomegaly, IgE, IFN-γ, IL-12, TNF-α, spontaneous proliferation	[36, 108, 110]

Locus/gene	Chr	*Leishmania* species	Controlled trait	Reference
Lmr16	2	*L. major*	Spontaneous proliferation	[36]
Lmr11	3	*L. major*	IgE, IL-6	[36, 108]
Lmr9	4	*L. major*	IgE, IL-6	[36, 108]
Lmr3	5	*L. major*	Splenomegaly, hepatomegaly, skin lesions, IgE, IFN-γ, spontaneous proliferation	[111]
Lmr4	6	*L. major*	Skin lesions, IFN-γ	[111]
Lmrq1	6	*L. major*	Skin lesions	[112]
Lmr21	7	*L. major*	Skin lesions, IFN-γ	[92]
Lmr10	8	*L. major*	Skin lesions, splenomegaly, IgE	[36, 108]
Slc7a2	8	*L. major*	Killing of parasites by macrophages in vitro	[101]
Lmr2/Fli1	9	*L. major*	Lesion size, wound healing	[94]
Lmr17	9	*L. major*	IFN-γ, TNF-α	[36]
Lmr19	10	*L. major*	Spontaneous proliferation	[36]
Lmr5	10	*L. major*	Skin lesions, splenomegaly, parasite load in spleen, IgE, IFN-γ, IL-4, IL-12	[36, 92, 108, 111]
Lmr6	11	*L. major*	IL-4	[111]
Lmrq4	11	*L. major*	Skin lesions	[112]
Lmr15	11	*L. major*	Hepatomegaly, IFN-γ	[36, 110]
Lmr22	12	*L. major*	IL-4	[92]
bg/Lyst	13	*L. donovani*	Parasite numbers in spleen	[102]
Lmrq5	15	*L. major*	Skin lesions	[112]
Lmr23	16	*L. major*	IFN-γ	[92]
Lmr12	16	*L. major*	IgE, TNF-α, IL-4, spontaneous proliferation	[36, 108]
Dice1b	16	*L. major*	Skin lesions, parasite numbers in lesions, IL-4 expression in vitro	[113]
Lmr18	16	*L. major*	Proliferation	[36]
Lmr7	17	*L. major*	Spontaneous proliferation	[111]
H2	17	*L. donovani*	Parasite load in liver, spleen and bone marrow	[107, 109, 114]
		L. infantum	Parasite load in liver, spleen and bone marrow	[107]
		L. mexicana	Parasite numbers in liver and spleen	[115]
		L. major	Skin lesions	[116]
		L. amazonensis	Skin lesions	[117]
Lmr1	17	*L. major*	Lesion size	[118, 119]
Lmr13	18	*L. major*	Lesion size, parasite load in spleen, IgE, TNF-α	[36, 108, 110]
Lmr30	X	*L. major*	Lesion size	[120]

In our studies we worked with two species of *Leishmania*: *L. major* and *L. tropica*.

The main aspects of the human life-threatening VL caused by *L. donovani* can be studied in mice infected by *L. major* [121]. These studies have provided important insights into the response of the host to infection [122, 123]. Several early studies stated that susceptibility to *L. major* is determined by activation of different types of T-cells [124, 125]. IL-4 producing Th2 T-cells were associated with progression of the disease whereas higher activation of IFNγ producing Th1 T-cells led to more resistant phenotype. Later studies revealed that also other components of the immune system are important for response to *L. major*. IL-10 production by regulatory T cells may be the reason of non-curing phenotype in mouse strains with predominant Th1 response [126]. IL-12 produced by macrophages is important for stimulating of Th1 response [127]. Dendritic cells [128, 129], neutrophils [130, 131] and keratinocytes [132] are important either for Th1 or Th2 responses to infection. Interestingly, chimeric mice containing different combinations of cells from a resistant and a susceptible strain provided evidence that both T-cell and non-T cell compartments are sufficient to confer a cure, and that T cells of a non-curing genotype can mediate cure in a 'curing environment' [133].

L. tropica causes CL but relatively recently was discovered that it can also visceralize [134]. It was revealed that systemic illness in veterans returning from the Middle East is in fact VL caused by *L. tropica* [135]. This has led to the investigation of less typical symptoms caused by this parasite. It was discovered, that *L. tropica* is responsible for VL in Kenya [136] and India [137, 138]. In Iran *L. tropica* caused VL [139], disseminated CL accompanied with VL [140] and also ML [141]. The reasons of this variability are not known but important may be genetic background of the patients. The genetic studies of the host were complicated because golden hamsters (*Mesocricetus auratus*) were considered as the best model of this disease and those are not inbred. Fortunately it was shown that strains of *L. tropica* from Afghanistan, India [142], and Turkey [143] cause CL in inbred BALB/c mice. Experiment with mice of the strains C57BL/6J, C57BL/10SgSnAi and gene-deficient mice on their backgrounds showed role of IL-10 and TGFβ in regulation of

parasite numbers in ears of infected mice [144]. *L. tropica* infection was also studied in CcS/Dem strains . These experiments revealed a strong effect of the host genotype on the course of the *L. tropica* infection. Females of strain CcS-16 developed the largest skin lesions and exhibited a unique systemic chemokine reaction, characterized by additional transient early peaks of CCL3 and CCL5, which was not present in CcS-16 males, other tested RCS and parental strains BALB/cHeA and STS [145].

2 Thesis aims

Aim of the thesis is to establish suitable mouse models for mapping of loci controlling infection caused by parasites of genera *Trypanosoma* and *Leishmania* and to use the results to distinguish the species-specific from the general genes controlling pathogenesis of diseases caused by the tested genera.

a) Establishment of a suitable mouse model for mapping of loci that control susceptibility to *T. b. brucei* infection. This will be achieved by testing parental strains BALB/cHeA and STS and RCS of the CcS/Dem series and by mapping *T. b. brucei* controlling loci in a strain that exhibits larger differences in susceptibility from BALB/c.

b) Mapping of *L. tropica* controlling loci in the highly susceptible RCS CcS-16.

c) To analyse eosinophil infiltration into inguinal lymph nodes of parental strains BALB/cHeA and STS and selected RCS from CcS/Dem series after *L. major* infection. To map loci controlling eosinophils infiltration into inguinal lymph nodes after *L. major* infection.

3 List of publications

Šíma, M., H. Havelková, L. Quan, M. Svobodová, T. Jarošíková, J. Vojtíšková, A. P. Stassen, P. Demant and M. Lipoldová (2011). Genetic control of resistance to *Trypanosoma brucei brucei* infection in mice. PLoS Neglected Tropical Diseases 5(6): e1173. **(IF_{2011}=4.716, citing articles without self-citations: 8)**

Sohrabi, Y., H. Havelková, T. Kobets, **M. Šíma**, V. Volkova, I. Grekov, T. Jarošíková, I. Kurey, J. Vojtíšková, M. Svobodová, P. Demant and M. Lipoldová (2013). Mapping the genes for susceptibility and response to *Leishmania tropica* in mouse. PLoS Neglected Tropical Diseases 7(7): e2282. **(IF_{2013}=4.489, citing articles without self-citations: 5)**

Slapničková, M., V. Volkova, M. Čepičková, T. Kobets, **M. Šíma**, M. Svobodová, P. Demant and M. Lipoldová (2016). Gene-specific sex effects on eosinophil infiltration in leishmaniasis. Biology of Sex Differences 7: 59. **(IF_{2016}=3.635, citing articles without self-citations: 2)**

Šíma, M., L. Kocandová, M. Lipoldová (2015). Genotyping of short tandem repeats (STRs) markers with 6 bp or higher length difference using PCR and high resolution agarose electrophoresis. Protocol Exchange doi:10.1038/protex.2015.054. **(IF=without IF, technical report)**

Other publishing activity not directly connected with the topic of this thesis:

Grekov I., A. Pombinho, **M. Šíma**, T. Kobets, P. Bartůňek, M. Lipoldová. Pharmaceutical composition comprising diphenyleneiodonium for treating diseases caused by the parasites belonging to the family *Trypanosomatidae*. Patent no. 305247, Awarded June 15th 2015, Industrial Property Office of the Czech Republic. WO2015039638-A1; CZ201300729-A3. **(Patent)**

4 Results

4.1 Genetic control of resistance to *Trypanosoma brucei brucei* infection in mice

To establish a suitable mouse model for mapping loci controlling survival after *T. b. brucei* infection we tested survival after infection parental strains BALB/cHeA and STS and several selected RCS: CcS-5, CcS-11, CcS-16 and CcS-20. These strains were selected based on a fact that they carry STS genotype (donor strain) at the site of previously mapped trypanosome controlling loci (*Tir1-3*) [11, 63-66]. Though parental strains BALB/cHeA and STS were comparably susceptible and showed short survival after infection (up to 16 days), strain CcS-11 (containing 12.5% of the genome from STS on BALB/cHeA genetic background) exhibited even shorter survival time (all mice died within 10 days). The difference was more prominent in females.

For further characterization of susceptibility in the strains BALB/cHeA, STS and CcS-11 we measured splenomegaly, hepatomegaly and changes of body weight 10 days p.i. and serum levels of GM-CSF, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL7/MCP-3 and TNF-α 2 and 10 days p.i in female mice of these strains. We observed increase of spleen to body weight ratio, liver to body weight ratio and production of measured cytokines (except GM-CSF) and decrease of body weight of infected mice in comparison with non-infected controls. In comparison with BALB/cHeA infected mice, CcS-11 infected mice had smaller splenomegaly, hepatomegaly (we observed this difference also in non-infected controls) and production of CCL7/MCP-3. There was no significant difference between infected mice of strains STS a CcS-11 in any of the observed phenotypes.

To map *T. b. brucei* infection controlling loci we infected 169 F_2 hybrids between BALB/cHeA and CcS-11 strains and measured their survival time. After the experiment, we isolated DNA from mouse tails and performed genotyping using 14 microsatellite markers covering STS regions of CcS-11 strain. Using statistical analysis we revealed four

T. b. brucei controlling loci (*Tbbr1-4* from *T. b. brucei* response) on chromosomes 3, 7, 12 and 19. They represent the first definition of genetic loci controlling susceptibility to *T. b. brucei* infection. Loci *Tbbr1* and *Tbbr2* exhibited significant linkage only in female mice and have main effects on survival that are not dependent on or influenced by interaction with other genes (main effects). These loci have in CcS-11 an opposite effect on the studied trait. The homozygosity for the STS allele of *Tbbr1* determines about 4 days longer survival than homozygosity of the BALB/cHeA allele, whereas homozygosity for the STS allele of *Tbbr2* is associated with about three days shorter survival than the homozygosity of the BALB/cHeA allele. *Tbbr3* influences survival in interaction with *Tbbr4*. F_2 mice with homozygous BALB/cHeA alleles at *Tbbr3* and STS (SS) alleles at *Tbbr4* or homozygous for STS allele at *Tbbr3* and homozygous for BALB/cHeA alleles in *Tbbr4* have the shorter survival in comparison with other combinations of *Tbbr3* and *Tbbr4* STS and BALB/cHeA alleles.

Tbbr2 maps in CcS-11 to a rather short STS-derived region on proximal part of chromosome 12, with previously estimated length of 6 cM [92, 146]. In order to map this locus more precisely, we genotyped this region with 8 microsatellite markers and 4 SNPs. This led to precision mapping of *Tbbr2* to a region to a region with a maximal length of 2.93 Mb that contains 48 genes (according recent version of MGI database: http://www.informatics.jax.org/).

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PLoS NEGLECTED TROPICAL DISEASES

Genetic Control of Resistance to *Trypanosoma brucei brucei* Infection in Mice

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Abstract

Background: *Trypanosoma brucei brucei* infects livestock, with severe effects in horses and dogs. Mouse strains differ greatly in susceptibility to this parasite. However, no genes controlling these differences were mapped.

Methods: We studied the genetic control of survival after *T. b. brucei* infection using recombinant congenic (RC) strains, which have a high mapping power. Each RC strain of BALB/c-c-STS/A (CcS/Dem) series contains a different random subset of 12.5% genes from the parental "donor" strain STS/A and 87.5% genes from the "background" strain BALB/c. Although BALB/c and STS/A mice are similarly susceptible to *T. b. brucei*, the RC strain CcS-11 is more susceptible than either of them. We analyzed genetics of survival in *T. b. brucei*-infected F_2 hybrids between BALB/c and CcS-11. CcS-11 strain carries STS-derived segments on eight chromosomes. They were genotyped in the F_2 hybrid mice and their linkage with survival was tested by analysis of variance.

Results: We mapped four *Tbbr* (*Trypanosoma brucei brucei* response) loci that influence survival after *T. b. brucei* infection. *Tbbr1* (chromosome 3) and *Tbbr2* (chromosome 12) have effects on survival independent of inter-genic interactions (main effects). *Tbbr3* (chromosome 7) influences survival in interaction with *Tbbr4* (chromosome 19). *Tbbr2* is located on a segment 2.15 Mb short that contains only 26 genes.

Conclusion: This study presents the first identification of chromosomal loci controlling susceptibility to *T. b. brucei* infection. While mapping in F_2 hybrids of inbred strains usually has a precision of 40–80 Mb, in RC strains we mapped *Tbbr2* to a 2.15 Mb segment containing only 26 genes, which will enable an effective search for the candidate gene. Definition of susceptibility genes will improve the understanding of pathways and genetic diversity underlying the disease and may result in new strategies to overcome the active subversion of the immune system by *T. b. brucei*.

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Introduction

Sleeping sickness (African trypanosomiasis) continues to pose a major threat to 60 million people in 36 countries in sub-Saharan Africa. The estimated number of new cases is currently between 50 000 and 70 000 per year (WHO 2006 – African trypanosomiasis - http://www.who.int/mediacentre/factsheets/fs259/en/). The disease is caused by infection with the tsetse fly-transmitted [1] protozoan haemoflagellate *Trypanosoma brucei*, which has three major sub-species: *T. b. gambiense*, *T. b. rhodesiense* and *T. b. brucei*. Two of them, *T. b. gambiense* and *T. b. rhodesiense* cause sleeping sickness in humans and can also infect animals; thus domestic and wild animals are an important parasite reservoir (WHO 2006 - http://www.who.int/mediacentre/factsheets/fs259/en/). The third species, *T. b. brucei* infects a wide range of mammals, but is unable to infect humans because it lacks the SRA (serum resistance-associated) protein that prevents lysis induced by Apolipoprotein L1, which is present in normal human serum [2,3]. *T. b. equiperdum* and *T. b. evansi*, which are derived from *T. b. brucei*, are adapted to transmission without development in tsetse fly, allowing these parasites to spread outside the African tsetse belt [4].

Upon the bite of the mammalian host by trypanosome-infected tsetse fly (*Glossina ssp.*), the parasites multiply locally in the skin and elicit a local host response in the form of an indurated skin lesion called the chancre. Eventually, the parasites enter the blood circulation via lymph vessels and can survive in the blood circulation throughout the infection of the host (reviewed in

Author Summary

Trypanosoma brucei are extracellular protozoa transmitted to mammalian host by the tsetse fly. They developed several mechanisms that subvert host's immune defenses. Therefore analysis of genes affecting host's resistance to infection can reveal critical aspects of host-parasite interactions. *Trypanosoma brucei brucei* infects many animal species including livestock, with particularly severe effects in horses and dogs. Mouse strains differ greatly in susceptibility to *T. b. brucei*. However, genes controlling susceptibility to this parasite have not been mapped. We analyzed the genetic control of survival after *T. b. brucei* infection using CcS/Dem recombinant congenic (RC) strains, each of which contains a different random set of 12.5% genes of their donor parental strain STS/A on the BALB/c genetic background. The RC strain CcS-11 is even more susceptible to parasites than BALB/c or STS/A. In F_2 hybrids between BALB/c and CcS-11 we detected and mapped four loci, *Tbbr1-4* (*Trypanosoma brucei brucei* response 1–4), that control survival after *T. b. brucei* infection. *Tbbr1* (chromosome 3) and *Tbbr2* (chromosome 12) have independent effects, *Tbbr3* (chromosome 7) and *Tbbr4* (chromosome 19) were detected by their mutual inter-genic interaction. *Tbbr2* was precision mapped to a segment of 2.15 Mb that contains 26 genes.

[5,6]), remaining continually exposed to the host's immune system. *T. brucei* species have the ability to penetrate the walls of capillaries and invade interstitial tissues, but they always remain extracellular as opposed to *T. cruzi* [6]. During the meningo-encephalitic phase of the infection parasites pass into brain where they cause serious pathology [7].

African trypanosomes have evolved very sophisticated evasion mechanisms to survive in chronically infected host. These evasion mechanisms include antigenic variation of the variant surface glycoprotein (VSG) [8] and the induction of alterations in the host's defense system, such as excessive activation of the complement system leading to persistent hypocomplementemia [9], anemia, thrombocytopenia [9], down regulation of nitric oxide production [10], polyclonal B-lymphocyte activation [11], and marked immunosuppression [12,13]. Most likely African trypanosomes induce also other, yet undiscovered, changes in the physiology of the infected host, which might interfere with effective control of the parasite [6].

Due to genetic and biological relatedness of *T. b. brucei* to other *Trypanosoma* species, many host responses to their infections are shared and therefore many aspects of human African trypanosomiasis (HAT) as well as livestock and horses infections are studied in experimental mouse infection with *T. b. brucei*. These experiments revealed great genetic variability among mouse strains in response to *T. b. brucei*, however not all results can be compared with each other because they were obtained in different experimental conditions using different *T. b. brucei* isolates. Strains DBA/2, BALB/c, BALB.B, and C3H/He are susceptible to *T. b. brucei* and display higher parasitemia, survive for a shorter time, whereas strains C57BL/10, C57BL/6, and B10.D2 are relatively resistant and survive a longer time [14,15]. In another experiment BALB/c mice exhibited higher parasitemia than C57BL/6, but they did not differ in survival [16]. Comparison of C57BL/6 and 129/SvEv showed that 129/SvEv exhibited higher parasitemia and lower specific IgM (but not IgG) antibody levels than C57BL/6 mice. Parasitemia was higher in 129Sv/Ev, but the weight loss, mortality and the number of trypanosomes in brain was higher in C57BL/6 [7]. CBA/N mice, deficient in production of a thymus-dependent high affinity antibody subset [17] survived longer than the strains CBA/CaT6 and A/J and had slightly lower splenomegaly, but all three strains exhibited similar numbers of circulating parasites [18].

Mouse genes controlling susceptibility to trypanosomiasis caused by the subgenus *T. (Nannomonas) congolense* [19–22] and by sub-genus *T. (Schizotrypanum) cruzi* the causative agent of Chagas disease [23], have been successfully mapped, but a genome-wide search for susceptibility loci to the subgenus *T. (Trypanozoon) brucei* has not yet been attempted.

We have therefore analyzed the genetic control of *T. b. brucei* resistance using the recombinant congenic (RC) strains of the BALB/c-c-STS/Dem (CcS/Dem) series. This series comprises 20 homozygous strains all derived from two parental inbred strains: the "background" strain BALB/c and the "donor" strain STS. Each CcS/Dem strain contains a different, random set of approximately 12.5% genes of the donor strain STS and approximately 87.5% genes of the background strain BALB/c [24]. This series has been successfully used to study genetics of complex diseases (partly reviewed in van Wezel et al. 2001 [25]), including infection with *Leishmania major* [26–30] and *Bordetella pertussis* [31].

In the present work, we show that RC strain CcS-11 differs in survival from both parental strains BALB/c and STS. In the cross between BALB/c and CcS-11, we mapped four genetic loci that influence survival after *T. b. brucei* infection. Two of these loci have individual effects; the other two operate in mutual non-additive interaction. This is the first report of genetic loci controlling resistance to *T. b. brucei*.

Materials and Methods

Mice

Mice of strains tested for survival BALB/cHeA (BALB/c) (10 females, 10 males), STS/A (10 females, 10 males), CcS-5 (10 females, 10 males), CcS-11 (10 females, 10 males), CcS-16 (9 females, 9 males) and CcS-20 (10 females, 10 males) were 13 to 23 weeks old (mean 17, median 17) at the time of infection. Splenomegaly, hepatomegaly, body weight changes and serum levels of seven cytokines and chemokines were analyzed using females of BALB/c (22 infected, 22 non-infected), STS (17 infected, 13 non-infected) and CcS-11 (25 infected, 26 non-infected), which were 8 to 19 week old (mean 13, median 13) at the time of infection. When used for these experiments, CcS/Dem strains passed more then 38 generation of inbreeding and therefore were highly homozygous. The regions of RCS' genomes inherited from the BALB/c or STS parents were defined [32]. 169 F_2 hybrids between CcS-11 and BALB/c (age 22 and 23 weeks at the time of infection) were produced at the Institute of Molecular Genetics. They comprised 85 females and 84 males and were tested simultaneously as a single experimental group. During the experiment, mice were placed into individually ventilated cages behind a barrier. The research had complied with all relevant European Union guidelines for work with animals and was approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics AS CR and by Departmental Expert Committee for the Approval of Projects of Experiments on Animals of the Academy of Sciences of the Czech Republic.

Parasites

The strain of *Trypanosoma brucei brucei* (AnTar1) was a generous gift of Jan van den Abbeele, Institute of Tropical Medicine "Prince Leopold", Antwerp, Belgium. Parasites stored in liquid nitrogen were thawed and used to infect BALB/c males by intraperitoneal

inoculation. Four to five days after infection, 10 µl of tail blood was collected, diluted in 90 µl of 1% formaldehyde in PBS, and the trypanosomes were counted in a Bürker counting chamber. Subsequently, tail blood was diluted in RPMI containing L-glutamine, sodium bicarbonate and glucose (Cat. Nr. R8758, Sigma, St. Louis, MO) in order to contain appropriate numbers of parasites for inoculation (Please see below).

Trypanosomiasis challenge

Mice were inoculated intraperitoneally with 2.5×10^4 bloodstream forms of *T. b. brucei* (AnTar1 strain) in 50. µl of RPMI containing L-glutamine, sodium bicarbonate and glucose (Cat. Nr. R8758, Sigma, St. Louis, MO). Survival time was measured in days following the day of challenge (day 0).

Disease phenotype

In the mice infected with *T. b. brucei*, 90 µl of blood were obtained 2 days after infection for determination of cytokine and chemokine levels. Mice were killed 10 days after inoculation. The blood, spleen, and liver were collected for the further analysis.

Cytokine and chemokine levels

Levels of GM-CSF (granulocyte-macrophage colony-stimulating factor), CCL2 (chemokine (C-C motif) ligand 2)/MCP-1 (monocyte chemotactic protein-1), CCL3/MIP-1α (macrophage inflammatory protein-1α), CCL4/MIP-1β (macrophage inflammatory protein 1-β), CCL5/RANTES (regulated upon activation, normal T-cell expressed, and secreted), CCL7/MCP-3 (monocyte chemotactic protein-3) and TNF-α, in serum were determined using Mouse chemokine 6-plex kit (Bender MedSystems, Vienna, Austria) and Mouse TNF-α simplex kit as multiplex assay. 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, CCL4/MIP-1β and TNF-α 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, Billerica, MA, USA) according to the protocol of Bender MedSystem. Beads were analyzed on flow cytometer LSR II (BD Biosciences, San Jose, CA, USA). Concentrations of cytokines were determined by Flow Cytomix Pro 2.4 software. 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, TNF-α2.1 pg/ml respectively.

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 regions on eight chromosomes [32]. These differential regions were typed in the F_2 hybrid mice between CcS-11 and BALB/c using 14 microsatellite markers (Research Genetics, Huntsville, AL, and Generi Biotech, Hradec Králové, Czech Republic): D1Mit403, D3Mit45, D7Mit25, D7Mit18, D7Mit282, D7Mit259, D8Mit85, D10Mit46, D10Mit12, D12Mit37, D16Mit73, D19Mit51, D19Mit60 and D19Mit46 (Table S1). The average distance between any two markers in the chromosomal segments derived from the strain STS or from the nearest BALB/c derived markers was 8.7 cM. 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, 1.5 mM $MgCl_2$ (except marker D7Mit259, for which the optimal concentration was 2.5 mM), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 0.5 U of Perfect Taq Red Polymerase (Central European Biosystems, Prague, Czech Republic) and approximately 40 ng of tail DNA. PCR reaction was performed using the DNA Engine Dyad® Peltier Thermal Cycler (Bio-Rad, Hercules, CA), 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 marker D7Mit259, for which optimal Ta = 52°C), 72°C for 60 s for elongation, and finally 3 min at 72°C for final extension. Each PCR product was electrophoresed in 3% agarose gel containing 80% of MetaPhor® Agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME) and 20% of UltraPure™ Agarose (Invitrogen, Carlsbad, CA) for 15 min to 2 h at 150 V.

Precision mapping of *Tbbr2*

To map precisely *Tbbr2* on STS derived segment of strain CcS-11 on proximal part of chromosome 12 [32] we used 8 microsatellite markers: D12Mit10a, D12Mit11, D12Mit209, D12Mit182, D12Mit104, D12Mit240, D12Mit170, *Dtnb* (dystrobrevin, beta) and 4 SNPs; rs48212577, rs4229232, rs50154157 and rs50776991 (Generi Biotech, Hradec Králové, Czech Republic). The conditions of PCR reaction were as described in the section Genotyping of F_2 mice.

Polymorphism of SNPs was tested by restriction analysis after PCR reaction using following restriction enzymes (New England BioLabs, Ipswich, MA): HpyAV for rs48212577 (14,13 µl of PCR product, 2 U (1 µl) of HpyAV, 1.7 µl of 10x NEB buffer 4 [200 mM Tris-acetate, 500 mM Potassium Acetate, 100 mM Magnesium Acetate, 10 mM Dithiothreitol, pH 7.9], 0.17 µl of 10 mg/ml BSA (bovine serum albumin), 37°C, o/n); HinfI for rs4229232 (14.8 µl of PCR product, 5 U (0,5 µl) of HinfI, 1.7 µl of 10x NEB buffer 4, 37°C, o/n); BsmFI for rs50154157 (14,13 µl of PCR product, 2 U (1 µl) of BsmFI, 1.7 µl of 10x NEB buffer 4, 0.17 µl of 10 mg/ml BSA, 65°C, o/n), and Tsp509I for rs50776991 (14,8 µl of PCR product, 2 U (0,5 µl) of Tsp509I, 1.7 µl of 10x NEB buffer 1 [100 mM Bis-Tris-propane-HCl, 100 mM $MgCl_2$, 10 mM Dithiothreitol, pH 7.0], 65°C, o/n). The products were electrophoresed in 3% agarose gel containing 80% of MetaPhor® Agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME) and 20% of UltraPure™ Agarose (Invitrogen, Carlsbad, CA) for 15 min to 2 h at 150 V.

Statistical analysis

For the strain pattern analyses, differences in survival after *T. b. brucei* infection were compared between the RC strains CcS-5, CcS-11, CcS-16 and CcS-20 and the parental strains BALB/c and STS by Kaplan-Meier estimator using the PROC LIFETEST procedure of the SAS 9.1 statistical package for Windows (SAS Institute, Inc., Cary, NC). The differences between strains BALB/c, STS and CcS-11 in splenomegaly, hepatomegaly and body weight change 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. Differences between strains BALB/c, STS and CcS-11 in chemokine and cytokine levels were calculated by Mann Whitney U test.

Linkage of microsatellite markers with survival after *T. b. brucei* infection in F_2 hybrids was examined by analysis of variance (ANOVA, PROC GLM statement of the SAS 8.2 for Windows (SAS Institute, Inc., Cary, NC)). Log_{10} transformation was performed in order to obtain normal distribution. The effect

of each marker, sex and experiment on mouse survival was tested. Each individual marker and its interactions with other markers and sex or experiment were subjected to ANOVA. A backward elimination procedure [33] was used. The first round of the backward elimination procedure results in a list of significant markers and a list of interactions. This list (the markers and interactions with P value smaller than 0.05) is the input for the second round of ANOVA and the marker (or interaction) bearing the highest P value (if $P>0.05$) is eliminated. The backward elimination procedure was repeated till the final set of significant markers and interactions was obtained.

To obtain genome-wide significance values (corrected P), the observed P-values (α_T) were adjusted according to Lander and Schork [34] using the formula:

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

where G = 1.75 Morgan (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); $\rho = 1.5$ for F_2 hybrids; h(T) = the observed statistic (F ratio).

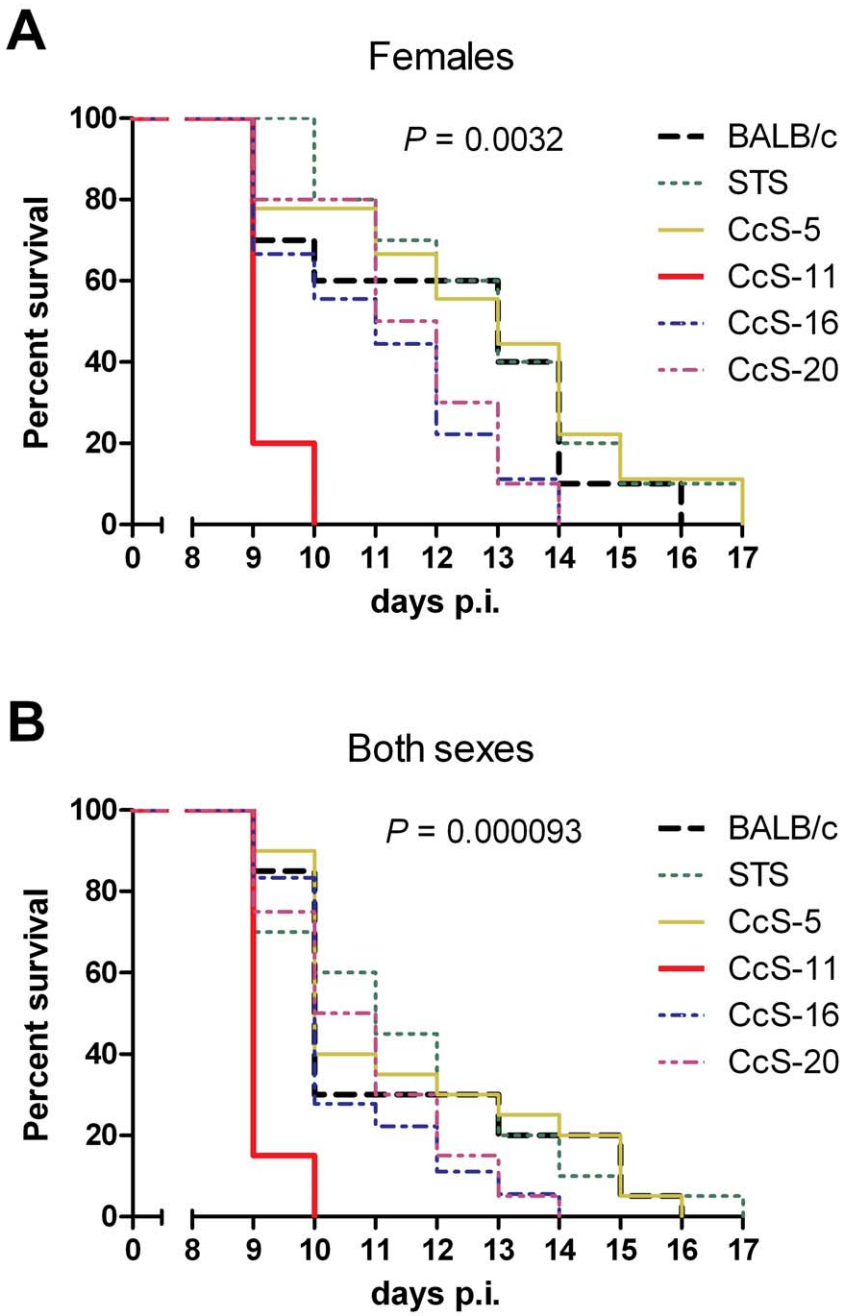

Figure 1. Differential survival of BALB/c, STS and selected RC strains after *T. b. brucei* infection. Survival of A. females or B. both sexes after intra-peritoneal inoculation of 2.5×10^4 bloodstream forms of *T. b. brucei*. 10 females and 10 males from each strain were used for experiment. The only exception was strain CcS-16, where we infected 9 females and 9 males.
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Results

Differences among mouse strains in survival after *T. b. brucei* infection

We have compared survival of strains BALB/c, STS/A, CcS-5, CcS-11, CcS-16 and CcS-20 after infection with *T. b. brucei*. Parental strains BALB/c and STS did not differ in survival. RC strains CcS-5, CcS-16, and CcS-20 did not significantly differ in survival from the background parental strain BALB/c. CcS-11 mice exhibit shorter survival than BALB/c mice after challenge with *T. b. brucei* infection ($P = 0.0032$ females, $P = 0.000093$ both sexes) (Figure 1 A,B). Some BALB/c mice survived up to 16 days, whereas none of the CcS-11 mice lived longer than 10 days. Strain CcS-11 was therefore selected for further analysis.

We have compared splenomegaly, hepatomegaly, changes of body weight (Figure 2), and differences in cytokine and chemokine

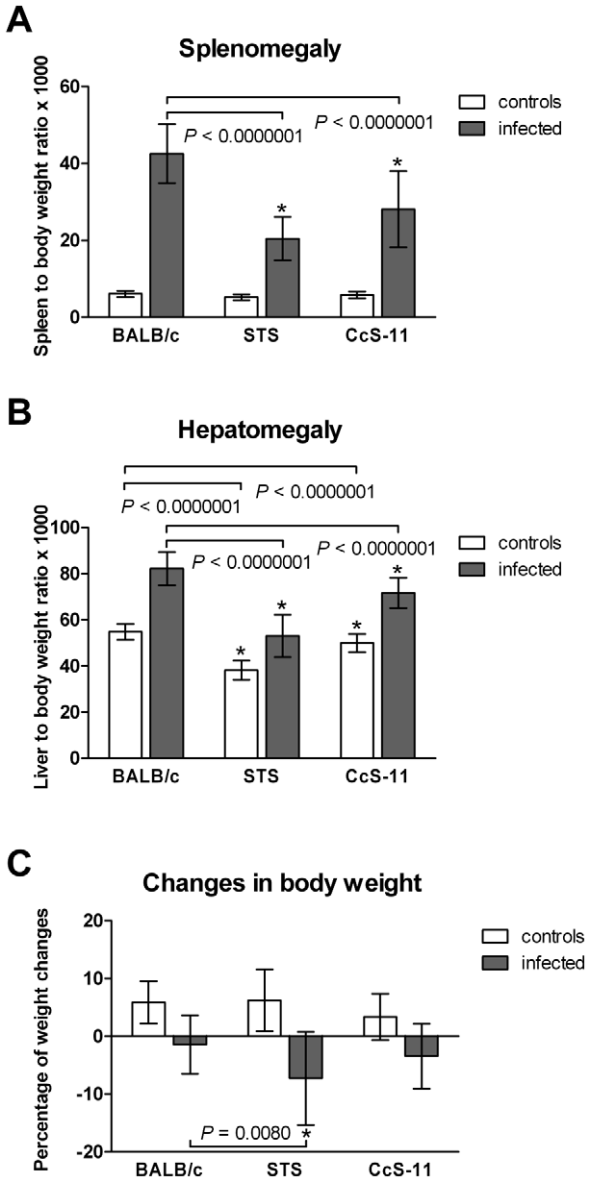

Figure 2. Differences in splenomegaly, hepatomegaly and changes in body weight after *T. b. brucei* infection. Female mice strains of BALB/c (17 infected, 16 non-infected), STS (17 infected, 13 non-infected) and CcS-11 (18 infected, 16 non-infected) were compared. Animals were intra-peritoneally inoculated with 2.5×10^4 bloodstream forms of *T. b brucei*. Control, non-infected mice were kept in the same animal facility. Both groups were killed after 10 days of infection. The data show the means ± SD from three independent experiments. Asterisks indicate significant difference from BALB/c.
doi:10.1371/journal.pntd.0001173.g002

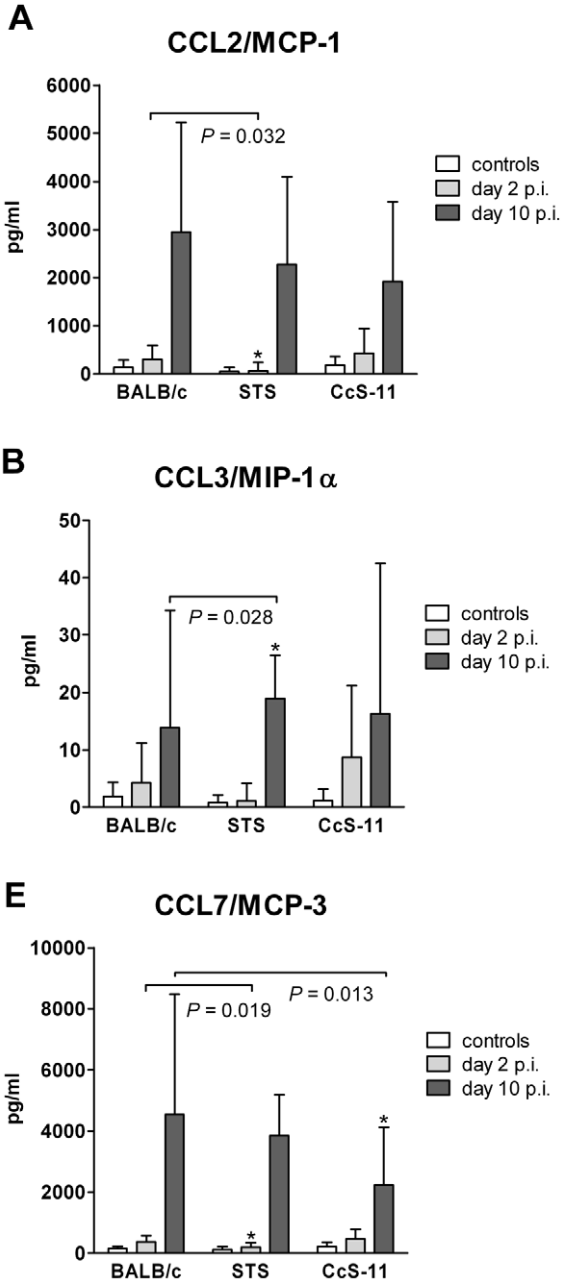

Figure 3. Differences in chemokine levels in strains BALB/c, STS and CcS-11 after *T. b. brucei* infection. Female mice strains of BALB/c (11 infected tested 2nd day p.i., 22 infected tested 10th day p.i., 22 non-infected), STS (9 infected tested 2nd day p.i., 17 infected tested 10th day, 13 non-infected) and CcS-11 (14 infected tested 2nd day p.i., 25 infected tested 10th day p.i., 26 non-infected) were compared. Animals were intra-peritoneally inoculated with 2.5×10^4 bloodstream forms of *T. b. brucei*. Control, non-infected mice were kept in the same animal facility. Mice were killed 10 days after inoculation. The data show the means ± SD from three independent experiments. Asterisks indicate significant difference from BALB/c.
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levels (Figure 3) in females of background strain BALB/c, donor strain STS and RC strain CcS-11. Non-infected mice do not differ in spleen to body weight ratio (Figure 2A) and in changes of body weight (Figure 2C), whereas liver to body weight was higher in BALB/c than in both STS ($P<0.0000001$) and CcS-11 ($P<0.0000001$) (Figure 2B). Infection led to a significant enlargement of spleens (BALB/c: $P=0.000001$; STS: $P=0.000004$; CcS-11: $P=0.000001$) and livers (BALB/c: $P=0.000001$; STS: $P=0.0007$; CcS-11: $P=0.000001$) in all tested strains and to decrease of body weight (BALB/c: $P=0.00068$; STS: $P=0.000044$; CcS-11: $P=0.00037$) in comparison with non-infected mice. BALB/c exhibited higher splenomegaly than STS ($P<0.0000001$) and CcS-11 ($P<0.0000001$) and also higher hepatomegaly than both STS ($P<0.0000001$) and CcS-11 ($P<0.0000001$). Differences in changes in body weight during the infection were observed between BALB/c and STS ($P=0.0080$).

Serum levels of CCL7/MCP-3, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, GM-CSF and TNF-α were measured at day 2 and 10 p.i. and compared with cytokines and chemokines serum levels of non-infected control mice. We did not observe any differences in GM-CSF levels between infected and non-infected mice. At day 2 p.i. all tested strains had increased levels of CCL7/MCP-3 in comparison with controls and in STS was also observed increased level of CCL5/RANTES. At day 10 p.i. all three tested strains exhibited increase of CCL7/MCP-3, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and TNF-α (Table S2, Figure 3, Figure S1). In infected mice, strain differences from BALB/c were observed in serum levels of CCL2/MCP-1, CCL3/MIP-1α and CCL7/MCP-3 (Figure 3). STS mice had lower serum level of CCL2/MCP-1 day 2 p.i. ($P=0.032$) (Figure 3A) and higher level of CCL3/MIP-1α day 10 p.i. ($P=0.028$) (Figure 3B) than BALB/c. STS mice had lower serum level of CCL7/MCP-3 than BALB/c day 2 p.i. ($P=0.019$), whereas CcS-11 had lower serum level of this chemokine than the background parental strain BALB/c day 10 p.i. ($P=0.013$) (Figure 3C).

Genetic loci that control survival after infection with *T. b. brucei*

We examined survival after *T. b. brucei* infection in 169 F_2 hybrids between the strains BALB/c and CcS-11. The strain CcS-11 differs from BALB/c in the genetic material at 8 chromosomes that were received from STS [32]. These differential STS-derived segments were genotyped in the F_2 hybrid mice using 14 microsatellite markers. Statistical analysis of linkage revealed four genetic loci that influence survival after *T. b. brucei* infection. Two of these loci have individual effects (Table 1); the other two operate in mutual non-additive interaction (Table 2). The effects of all loci were more expressed in females than in males.

Two loci, *Tbbr1* (*Trypanosoma brucei brucei* response 1) linked to D3Mit45 (corrected P value = 0.0494 females; corr. $P=0.267$ both sexes) and *Tbbr2* linked to D12Mit37 (corrected P value = 0.0224 females; corr. P value = 0.0583 both sexes) have main effects on survival that are not dependent on or influenced by interaction with other genes (main effects) (Table 1, Figure 4 A,B,C,D). These loci have in CcS-11 an opposite effect on the studied trait. The homozygosity for the STS allele of *Tbbr1* (SS) determines about 4 days longer survival than homozygosity of the BALB/c allele (CC), whereas homozygosity for the STS allele of *Tbbr2* (SS) is associated with about three days shorter survival than the homozygosity of the BALB/c allele (CC). We have also observed a suggestive linkage of survival to D8Mit85 (corrected P value = 0.0542 females; corr. $P=0.0994$ both sexes), heterozygotes had the shorter survival (Table 1).

Tbbr3 linked to D7Mit282 influences survival in interaction with *Tbbr4* linked to D19Mit51 (corrected $P=0.0332$ females; corr. $P=0.0430$ both sexes). F_2 mice with homozygous BALB/c (CC) alleles at *Tbbr3* and STS (SS) alleles at *Tbbr4* or homozygous for STS allele at *Tbbr3* and homozygous for BALB/c alleles in *Tbbr4* have the shorter survival in comparison with other combinations of *Tbbr3* and *Tbbr4* STS and BALB/c alleles (Table 2, Figure 4 E,F). A suggestive linkage was detected in females in interaction of D8Mit85 and D19Mit60 (corrected $P=0.0555$), shorter survival has been observed in mice heterozygous both in D8Mit85 and D19Mit60 (Table 2).

Precision mapping of *Tbbr2*

Tbbr2 maps in CcS-11 to a rather short STS-derived region on proximal part of chromosome 12, with previously estimated length of 6 cM [32,35]. In order to map this locus more precisely, we genotyped this region with 8 microsatellite markers and 4 SNPs. This led to precision mapping of *Tbbr2* to a region with a maximal length of 2.15 Mb that contains only 26 genes (Figure 5).

Discussion

CcS-11 differs in susceptibility to trypanosomiasis from both parental strains

CcS-11 differs in susceptibility to trypanosomiasis from both parental strains. The background strain BALB/c is susceptible to

Table 1. Loci controlling survival after *T. b. brucei* infection - Single gene effect.

Marker	Group	Genotype						*P* value	corr. *P* value
		CC		CS		SS			
D3Mit45	females	15.88±0.36	(n = 18)	16.98±0.41	(n = 48)	19.58±0.93	(n = 19)	0.0010	**0.0494**
(*Tbbr1*)	both sexes	15.17±0.35	(n = 39)	15.73±0.33	(n = 96)	17.22±0.40	(n = 34)	0.0077	NS (0.267)
D8Mit85	females	18.32±0.86	(n = 20)	15.99±0.38	(n = 44)	18.03±0.85	(n = 20)	0.0011	0.0542 (suggestive)
	both sexes	16.98±0.40	(n = 43)	15.14±0.35	(n = 89)	15.99±0.37	(n = 36)	0.0024	NS (0.0994)
D12Mit37	females	19.23±0.90	(n = 15)	17.29±0.43	(n = 53)	15.89±0.36	(n = 15)	0.0004	**0.0224**
(*Tbbr2*)	both sexes	17.06±0.40	(n = 32)	15.74±0.27	(n = 98)	15.38±0.35	(n = 37)	0.0013	0.0583 (suggestive)

F_2 hybrids between CcS-11 and BALB/c were tested. Means and standard errors of means of survival times and *P*-values were computed by analysis of variance. Logarithmic (Log_{10}) transformation was used to obtain normal distribution and the obtained values were retransformed after calculation. Number of tested mice is shown in brackets.

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Table 2. Interaction between loci that control survival after *T. b. brucei* infection.

A. Females				
		$P = 0.0009$		**Corrected $P = 0.0332$**
		D19Mit51 (*Tbbr4*)		
		CC	**CS**	**SS**
D7Mit282	**CC**	18.46±1.32	18.79±0.88	16.08±0.74
(*Tbrr3*)		(n = 6)	(n = 11)	(n = 9)
	CS	18.14±0.85	17.77±0.86	17.01±0.81
		(n = 9)	(n = 18)	(n = 9)
	SS	14.90±1.06	17.33±0.82	18.66±0.88
		(n = 5)	(n = 9)	(n = 6)
		$P = 0.0016$		**Corrected $P = 0.0555$**
		D19Mit60		
		CC	**CS**	**SS**
D8Mit85	**CC**	18.96±1.36	16.68±0.78	19.45±1.39
		(n = 5)	(n = 8)	(n = 7)
	CS	17.06±0.76	15.05±0.36	15.94±0.77
		(n = 10)	(n = 23)	(n = 11)
	SS	16.96±1.20	20.13±1.44	17.16±0.78
		(n = 5)	(n = 7)	(n = 8)
B. Both sexes				
		$P = 0.0013$		**Corrected $P = 0.0430$**
		D19Mit51 (*Tbbr4*)		
		CC	**CS**	**SS**
D7Mit282	**CC**	16.63±0.79	17.02±0.40	15.24±0.72
(*Tbbr3*)		(n = 11)	(n = 19)	(n = 12)
	CS	16.44±0.79	16.21±0.39	15.70±0.37
		(n = 19)	(n = 40)	(n = 24)
	SS	13.99±0.67	16.48±0.38	16.90±0.80
		(n = 9)	(n = 22)	(n = 10)
		$P = 0.0420$		**Corrected P = NS**
		D19Mit60		
		CC	**CS**	**SS**
D8Mit85	**CC**	18.20±0.85	16.11±0.75	16.90±0.80
		(n = 8)	(n = 18)	(n = 17)
	CS	15.92±0.31	14.59±0.33	14.96±0.35
		(n = 20)	(n = 49)	(n = 20)
	SS	15.84±0.76	16.90±0.80	15.24±0.72
		(n = 9)	(n = 16)	(n = 11)

F_2 hybrids between CcS-11 and BALB/c were tested. Means and standard errors of means of survival times and *P*-values were computed by analysis of variance. Logarithmic (Log_{10}) transformation was used to obtain normal distribution and the obtained values were retransformed after calculation. Number of tested mice is shown in brackets.

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T. b. brucei. This is in agreement with findings of other research groups [15,16]. Donor strain STS does not differ in survival from the background strain BALB/c, however the strain CcS-11 that contains a set of approximately 12.5% genes of the donor strain STS and 87.5% genes of the background strain BALB/c and it has shorter survival after infection than either parent. The elements in the BALB/c genome that work in interaction with STS disease response loci can be identified in linkage tests as gene-gene interactions. For example, in the interaction of *Tbbr3* and *Tbbr4*, the survival of mice with homozygous BALB/c alleles at both loci, or homozygous STS alleles at both loci is longer than of mice that are homozygous for BALB/c allele at one locus and homozygous for the STS allele at the second (Table 2A). The fine mapping and molecular identification of *Tbbr4* will reveal one of BALB/c elements that can modify the effect of STS genes. The RC strains are especially suitable to detect such interactions [36].

The observations of progeny having a phenotype, which is beyond the range of the phenotype of its parents are not rare in traits controlled by multiple genes. Some F_2 hybrids derived in cross between trypanotolerant African N'Dama (*Bos taurus*) and trypanosusceptible Kenya Boran (*Bos indicus*) cattle differed from both parents and contained less *T. congolense* parasites than any of them [37]. Similarly, mouse RC strain OcB-9 differs from both parental strains O20 and B10.O20 in response to alloantigens [38], several RC strains exhibit in some parameters higher susceptibility to *Leishmania major* than both parental strains BALB/c and STS [39], and analysis of gene expression from livers in chromosome substitution strains (background strain C57BL/6, donor strain A/J) revealed that only 438 out of 4209 expression QTLs were inside the parental range [40]. These observations are due to multiple gene-gene interactions of QTLs, which in new combinations of these genes in RC strains, F_2 hybrids or in chromosomal substitution strains can lead to appearance of new phenotypes that exceed their range in parental strains. Also, with traits controlled by multiple loci, the parental strains often contain susceptible alleles at some of them and resistant on others, and some progeny may receive predominantly susceptible alleles from both parents.

We have compared in strains BALB/c, STS and CcS-11 splenomegaly, hepatomegaly, changes of body weight (Figure 2), and cytokine and chemokine levels (Figure 3). However, none of these measurements explains differences in survival between BALB/c and CcS-11. BALB/c and CcS-11 also do not differ in parasitemia day 10 p.i. (data not shown). Thus, the identification of *Tbbr1-Tbbr4* genes is needed to provide information about the mechanisms controlling differences in survival between these strains.

Susceptibility loci and potential candidate genes

We have detected four loci that in the strain CcS-11 control survival after *T. b. brucei* infection and mapped them with a precision of 1 cM–25 cM (Tables 1, 2, Figure 5). Usually, a standard inbred-strain mapping experiment using F_2 hybrids will map a QTL onto a 20- to 40-cM interval [41]. Using advanced intercross lines [20,22] the susceptibility loci *Tir1* and *Tir3c* to *T. congolense* were mapped with a 95% confidence interval to 1.3 and 2.2 cM, respectively. In the RC strains the donor-derived segments of medium length (5–25 cM) comprise 54% of donor genome [42]. However, RC strains can carry on some chromosomes very short segments of donor strain origin. This feature of the RCS system allowed us previously to narrow the location of *Lmr9* (*Leishmania major* response 9) on chromosome 4 to a short segment of 1.9 cM without any additional crosses [43]. The short length of this segment, which controls levels of serum IgE in *L. major* infected mice, enabled us to map a human homolog of this locus on human chromosome 8 and show that it controls susceptibility to atopy [44].

Our data show sex influence on survival as after correction for the genome-wide testing significance of the *Tbbr* loci was detected only in females or in the whole tested group. This observation can be related to the influential role of sex hormones in control of parasitic infections by their ability to modulate different compo-

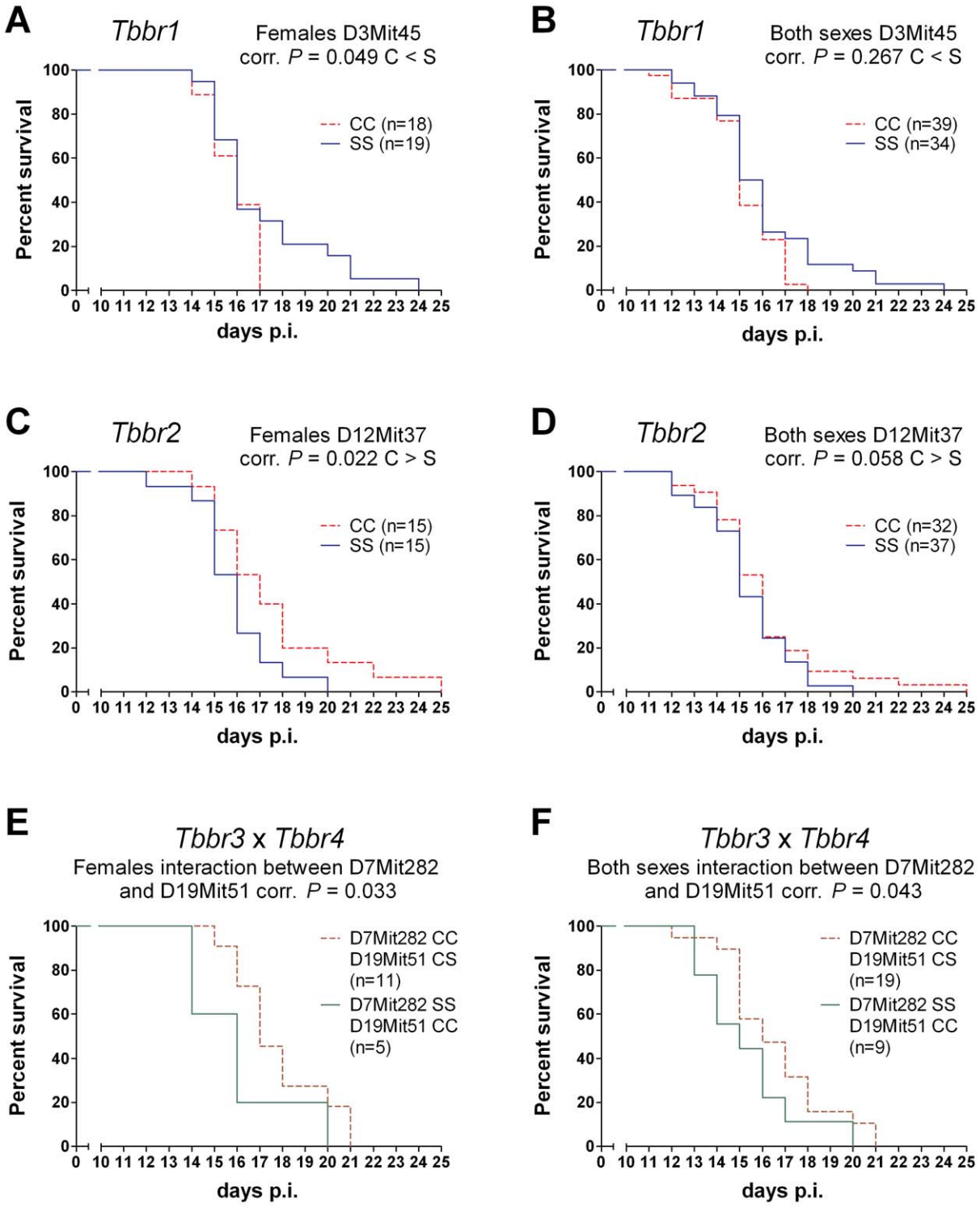

Figure 4. Differential survival of F_2 hybrid mice after *T. b. brucei* infection. Mice were intra-peritoneally inoculated by 2.5×10^4 bloodstream forms of *T. b. brucei*. **A.** females and **B.** both sexes carrying BALB/c or STS homozygous alleles in *Tbbr1* (D3Mit45); **C.** females and **D.** both sexes carrying BALB/c or STS homozygous alleles in *Tbbr2* (D12Mit37); **E.** females and **F.** both sexes carrying interacting STS homozygygous alleles in *Tbbr3* (D7Mit282) and BALB/c homozygous alleles in *Tbbr4* (D19Mit51) or BALB/c homozygous alleles in *Tbbr3* and heterozygotes in *Tbbr4*. n, number of mice.

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nents of both the innate and adaptive immune responses [45,46]. Greenblatt and Rosenstreich [47] analyzed resistance of the 10 inbred mouse strains and two sets of F_1 hybrids to infection with *T. b. rhodesiense*. C3H/HeN, C3H/HeJ, CBA/J, BALB/c and CBA/CaJ were highly susceptible, with mean survival times of less than 22 days, and did not exhibit differences in survival between males

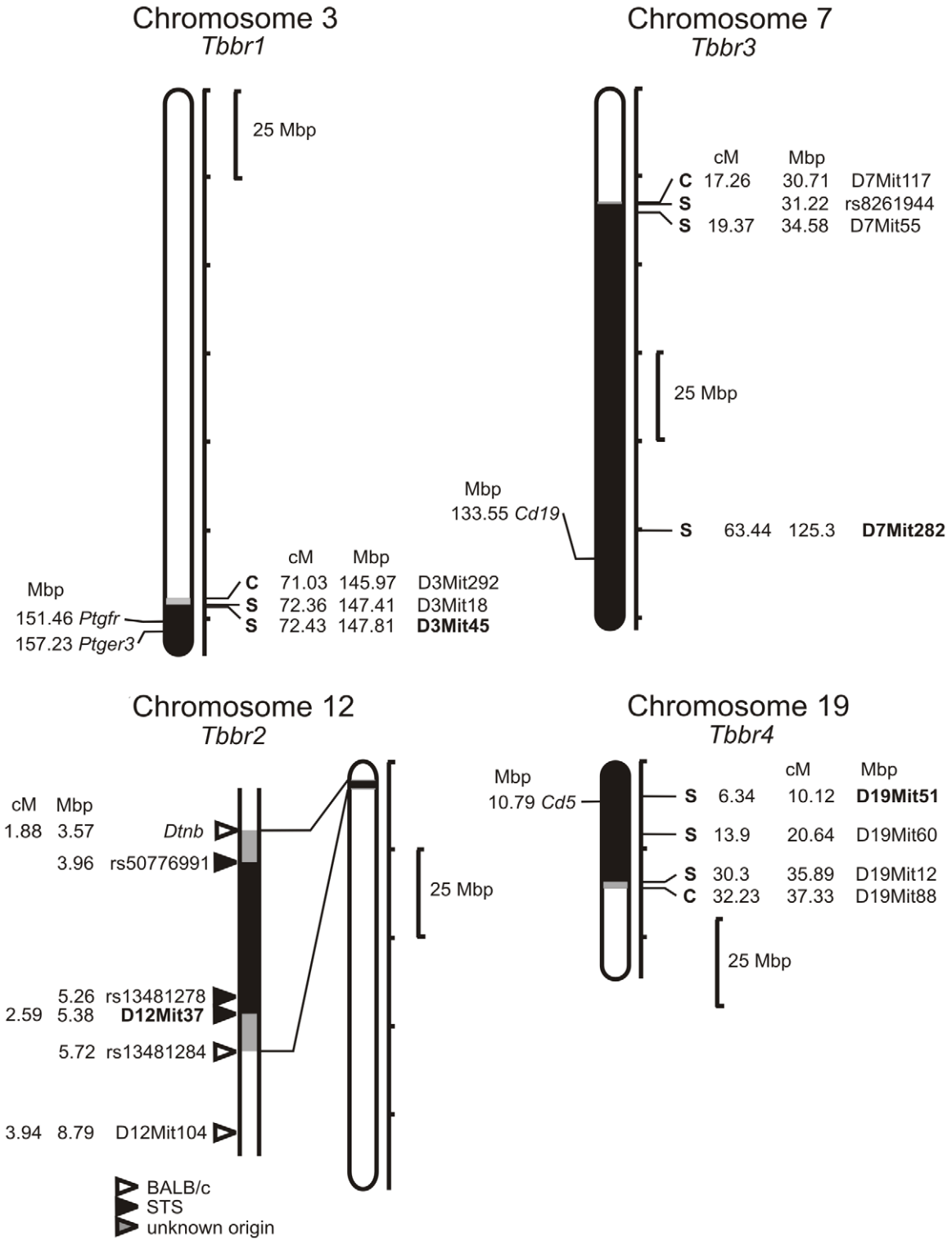

Figure 5. Position of the loci that control response to *T. b. brucei* in strain CcS-11. The regions of STS and BALB/c origin are represented as dark and white, respectively; the boundary regions of undetermined origin are shaded. Only the markers defining the boundaries the STS-derived segment and the markers that were tested for linkage are shown. The markers that exhibit significant *P* values (corrected for genome-wide search) are shown in bold.
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and females, whereas in more resistant strains CBA/N, A.CA, C57BL/6J, C57BL/KsJ, C57BL/10SnJ, (BALB/c x C57BL/6)F_1 and (C57BL/6× BALB/c)F_1 female mice were more resistant than males. These data support the finding of different genetic regulation of susceptibility to *T. brucei* in males and females in certain genetic combinations. 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 [48] and *Hrl* (herpes resistance locus) exhibits higher influence on susceptibility to Herpes simplex virus in male than in female mice [49]. 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, but it has an opposite effect on disease susceptibility in males and females [50]. *Lmr20* influenced IgE level in *L. major* infected females, but not in males [35]. QTLs *Cnes1* and *Cnes2* were associated with high pulmonary *Cryptococcus neoformans* burden in females, whereas *Cnes3* was associated with fungal pulmonary burden in male mice [51]. QTL on chromosome 17 controls susceptibility to pulmonary infection with *Chlamydia pneumoniae*, but has much stronger effect in males, whereas QTL on chromosome 5 controls susceptibility only in female mice [52]. In humans, for example the *IL9* genetic polymorphism (rs2069885) has an opposite effect on the risk of severe respiratory syncytial virus bronchiolitis in boys and girls [53].

In the present study, we were able to precision map *Tbbr2* to 2.15 Mb. This segment contains 26 genes, 12 of them are either predicted genes or cDNA sequences (Table 3). Public databases (http://www.ncbi.nlm.nih.gov; http://www.informatics.jax.org and http://biogps.gnf.org/#goto=welcome) show that some of these genes are in non-infected mice expressed in tissues such as liver, spleen, and brain (Table 3). These organs are in infected mice affected by parasite [6,7]. There is no obvious candidate gene and there are only indirect indications about the possible role of some of these genes, such as *Dnmt3a* (DNA methyltransferase 3a)[54],[55], *Pomc* (pro-opiomelanocortin-alpha) [56,57], *Adcy3* (adenylate cyclase 3) [58], and *Ncoa1* (nuclear receptor coactivator 1) [59] in immune response against *Trypanosoma*.

Tbbr1 is localized in the distal part of chromosome 3. Potential candidate genes in this locus are *Ptgfr* (prostaglandin F receptor) [MGI:97796] and *Ptger3* (prostaglandin E receptor 3 (subtype EP3)) [MGI:97795], as prostaglandins play a suppressive role in infection with African trypanosomes [60].

Tbbr3 on chromosome 7 and *Tbbr4* on chromosome 19 map near to the genes *Cd19* [MGI:88319] and *Cd5* [MGI:88340], respectively, that code markers of B lymphocytes. CD19 is a B-

Table 3. Expression of genes in locus *Tbbr2* in liver, spleen and brain of non-infected animals.

Gene	ID	Liver		Spleen		Brain	
		NCBI,MGI	BioGPS	NCBI,MGI	BioGPS	NCBI,MGI	BioGPS
Gm11061, predicted gene 11061	MGI:3779285	NT	NT	NT	NT	NT	NT
Dnmt3a, DNA methyltransferase 3A	MGI:1261827	**YES**	<<M	**YES**	**>M**	**YES**	**>M**
Gm10485, predicted gene 10485	MGI:3641689	NT	NT	NT	NT	NT	NT
Pomc, pro-opiomelanocortin-alpha	MGI:97742	NO	<<	NO	<<	**YES**	<<
Efr3b, EFR3 homolog B (S. Cerevisiae)	MGI:2444851	NO	**>M**	NO	**>M**	**YES**	**>M**
Dnajc27, Dnaj (Hsp40) homolog, subfamily C, member 27	MGI:2443036	NO	<M	NO	<M	**YES**	**>30xM**
Adcy3, adenylate cyclase 3	MGI:99675	**YES**	<M	**YES**	**>M**	**YES**	**>3M**
Cenpo, centromere protein O	MGI:1923800	**YES**	<M	NO	<M	**YES**	<M
2410017P09Rik, RIKEN cDNA 2410017P09 gene	MGI:1916959	**YES**	**>M**	NO	<M	**YES**	**>M**
Ncoa1, nuclear receptor coactivator 1	MGI:1276523	**YES**	<M	**YES**	<M	**YES**	**>3xM**
Gm3613, predicted gene 3613	MGI:3781789	NO	NT	NO	NT	NO	NT
Gm3620, predicted gene 3620	MGI:3781796	NO	**>M**	NO	**>M**	NO	**>M**
Gm3625, predicted gene 3625	MGI:3781801	NO	NT	**YES**	NT	**YES**	NT
Itsn2, intersectin 2	MGI:1338049	**YES**	<M	**YES**	**>3xM**	**YES**	**>M**
4930417G10Rik, RIKEN cDNA 4930417G10 gene	MGI:1922105	NO	**>M**	NO	<M	**YES**	**>M**
A830093I24Rik, RIKEN cDNA A830093I24 gene	MGI:2442121	**YES**	**>M***	NO	**>M***	**YES**	**>M***
Pfn4, profilin family member 4	MGI:1920121	NO	<M**	NO	NT	**YES**	<M**
Gm6682, predicted gene 6682	MGI:3647156	NT	**>M**	NT	<M	NT	<M
0610009D07Rik, RIKEN cDNA 0610009D07 gene	MGI:1913305	**YES**	<M	**YES**	**>M**	**YES**	<M
Fkbp1b, FK506 binding protein 1b	MGI:1336205	NO	<M	NO	<M	**YES**	<M
BC068281, cDNA sequence BC068281	MGI:3040699	**YES**	**>3xM**	**YES**	**>M**	**YES**	**>M**
Mfsd2b, major facilitator superfamily domain containing 2B	MGI:3583946	NO	<M	**YES**	<M	NO	<M
Ubxn2a, UBX domain protein 2A	MGI:2442310	**YES**	**>M**	**YES**	**>M**	**YES**	**>3M**
Atad2b, ATPase family, AAA domain containing 2B	MGI:2444798	**YES**	<M	**YES**	**>M**	**YES**	**>M**
Klhl29, kelch-like 29 (Drosophila)	MGI:2683857	NO	<M	NO	**>3M**	**YES**	**>30xM**
2810032G03Rik, RIKEN cDNA 2810032G03 gene	MGI:1919919	NO	**>M**	NO	<M	**YES**	**>3xM**

Data were compiled from public databases (Http://www.ncbi.nlm.nih.gov; http://www.informatics.jax.org) February 25, 2011 and http://biogps.gnf.org/#goto=welcome, February 25, 2011). NCBI/MGD: YES – expression of a gene was observed; NO – expression of a gene was not observed; NT – not tested. BioGPS: Majority of data were obtained using Gene Atlas MOE430, *Gene Atlas GNF1M, **Gene Atlas U133A. M = median value across all samples for a single probe set. NT – not tested.

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lineage antigen, present on both B-1 and B-2 cells [61]. It was shown that in murine experimental *T. brucei* trypanosomiasis, B-cells were crucial for periodic peak parasitemia clearance and survival of host [16]. $CD5^+$ subpopulation of B-1 cell has been found to be stimulated by different *Trypanosoma* species: *T. cruzi* [62], *T. b. evansi* [63], and *T. congolense* [64]. These B-cells were the main source of antibodies reactive with non-parasite antigens in *T. congolense*-infected cattle [64].

However, genes that are presently not considered as possible candidates might cause the effects of some or all *Tbbr* loci. Moreover, not only genes, but also noncoding RNAs in *Tbbr* loci region may influence the outcome of infection [65].

Are *Tbbr* loci involved in control of other pathogens?

Some genes, for example *Slc11a1* (solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1) or *Lyst* (lysosomal trafficking regulator) */beige* have been found to control susceptibility to several pathogens (reviewed in ref [29]). *Tbbr2* might be potentially involved also in control of *Leishmania major*, as it overlaps with locus *Lmr22 (Leishmania major* response 22*)*, which in interaction with *Lmr5* controls serum IL-4 in *L. major* infected mice [35], whereas *Tbbr3* on chromosome 7 maps near to *Ity7* (immunity to *S. typhimurium 7*) [66].

Control of susceptibility to *T. congolense* is exercised by loci on chromosomes 17, 5 and 1 [19,22], whereas susceptibility to *T. cruzi* is determined by loci on chromosomes 17 and 5 [23]. Influence of loci on chromosomes 17 and 5 could not be tested in the present cross, as CcS-11 does not carry STS-derived segments on these chromosomes [32]. STS-derived region present on chromosome 1 of CcS-11 overlaps with *Tir3c* [22], however we did not detect influence of this segment on susceptibility to *T. b. brucei*. This might be caused either by differences in regulation of immunity against the sub-genus *T. (Nannomonas) congolense* and the subgenus *T. (Trypanozoon) brucei*, or because the *Tir3c*, which was detected in a cross between strains C57BL/6J and BALB/c [19] and C57BL/6J and A/J [22] is not polymorphic between strains BALB/c and STS tested in this paper. Therefore the possible effects of *Tbbr* loci in infection with other *Trypanosoma* species have yet to be established.

In summary, this study represents the first definition of genetic loci controlling susceptibility to *T. b. brucei* infection. One of them, *Tbbr2* is precisely mapped to the segment that contains only 26 genes, which will facilitate the identification of the candidate gene.

T. brucei subspecies cause sleeping sickness in humans and affect also all livestock, with particularly severe effects in horses and dogs [1]. Thus, the definition of genes controlling anti-parasite responses might also permit a better understanding of pathways and genetic diversity underlying the disease phenotypes in humans and domestic animals.

Supporting Information

Figure S1 Differences in levels of CCL4/MIP-1β, CCL5/RANTES, and TNF-α between infected and non-infected mice. Female mice strains of BALB/c (11 infected tested 2nd day p.i., 22 infected tested 10th day p.i., 22 non-infected), STS (9 infected tested 2nd day p.i., 17 infected tested 10th day, 13 non-infected) and CcS-11 (14 infected tested 2nd day p.i., 25 infected tested 10th day p.i., 26 non-infected) were compared. Animals were intra-peritoneally inoculated with 2.5×10^4 bloodstream forms of *T. b. brucei*. Control, non-infected mice were kept in the same animal facility. Mice were killed 10 days after inoculation. The data show the means ± SD from three independent experiments.
(TIF)

Table S1 Survival times and genotypes of F_2 hybrids between BALB/c and CcS-11.
(XLS)

Table S2 *P* values of differences in serum chemokines and cytokines levels between non-infected and infected mice.
(DOC)

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Author Contributions

Conceived and designed the experiments: MŠ HH ML. Performed the experiments: MŠ HH MS TJ JV. Analyzed the data: MŠ LQ APMS PD ML. Wrote the paper: MŠ PD ML.

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4.2 Mapping the genes for susceptibility and response to *Leishmania tropica* in mouse

In order to map loci controlling response to *Leishmania tropica* we performed an experiment on 247 female F_2 hybrids between RCS CcS-16 (containing 12.5% of the genome from STS on BALB/cHeA genetic background) and parental strain BALB/cHeA. Females of the strain CcS-16 were chosen for mapping based on previous finding where CcS-16 females developed the largest lesions and exhibited an unique systemic chemokine reaction, characterized by additional transient early peaks of CCL3 and CCL5 in comparison with CcS-16 males, parental strains BALB/cHeA and STS and other tested RCS of CcS/Dem series [145]. We infected the mice with *L. tropica* and then every second week measured the size of the skin lesions and collected blood. We killed the mice 43 weeks after inoculation and collected blood, spleen, liver, inguinal lymph nodes and tails for later analysis. From tail samples we isolated DNA and performed genotyping using 23 microsatellite markers covering STS regions of CcS-16 strain. We measured parasite load in lymph nodes, spleen and liver samples using PCR-ELISA [147] and levels of GM-CSF, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES and CCL7/MCP-3 in sera collected 7 weeks p.i. Using statistical analysis we were able to map eight novel *L. tropica* controlling loci named *Ltr1-8* (from *L. tropica* response). These loci are with controlled phenotypes listed in Table 4.

Table 4. Loci controlling response to *L. tropica.*

Locus	chr.	Marker	Phenotype controlled
Ltr1	2	D2Mit156	parasites in lymph nodes (int. *Ltr4* - D4Mit153*)
Ltr2	2	D2Mit389, D2Nds3/*Il1b*, D2Mit257, D2Mit52	skin lesions week 19; skin lesions week 21; splenomegaly (int. *Ltr3* - D3Mit11*); parasites in liver; hepatomegaly; CCL7; CCL7 (int. *Ltr6* - D11Mit37*)
Ltr3	3	D3Mit25, D3Mit11	splenomegaly (int. *Ltr2* - D2Mit257*); parasites in spleen (transgenerational parental effect); CCL3; CCL3 (int. *Ltr7* - D17Mit130*); CCL5; CCL5 (int. *Ltr7* - D17Mit130*)
Ltr4	4	D4Mit153	parasites in lymph nodes (int. *Ltr1* - D2Mit156*); parasites in liver (int. *Ltr8* - D18Mit40*)
Ltr5	10	D10Mit67, D10Mit103	splenomegaly (int. *Ltr7* - D17Mit130*); splenomegaly (int. *Ltr8* - D18Mit49*)
Ltr6	11	D11Mit37	parasites in spleen (trangenerational parental effect); CCL7 (int. *Ltr2* - D2Mit257*)
Ltr7	17	D17Mit130	splenomegaly (int. *Ltr5* - D10Mit67*); CCL3 (int. *Ltr3* - D3Mit11*); CCL5 (int. *Ltr3* - D3Mit11*)
Ltr8	18	D18Mit40, D18Mit49	splenomegaly; splenomegaly (int. *Ltr5* - D10Mit103*); parasites in liver (int. *Ltr4* - D4Mit153*); CCL7

* locus controls given phenotype in interaction with another locus

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PLOS | NEGLECTED TROPICAL DISEASES

Mapping the Genes for Susceptibility and Response to *Leishmania tropica* in Mouse

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Abstract

Background: *L. tropica* can cause both cutaneous and visceral leishmaniasis in humans. Although the *L. tropica*-induced cutaneous disease has been long known, its potential to visceralize in humans was recognized only recently. As nothing is known about the genetics of host responses to this infection and their clinical impact, we developed an informative animal model. We described previously that the recombinant congenic strain CcS-16 carrying 12.5% genes from the resistant parental strain STS/A and 87.5% genes from the susceptible strain BALB/c is more susceptible to *L. tropica* than BALB/c. We used these strains to map and functionally characterize the gene-loci regulating the immune responses and pathology.

Methods: 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.

Principal Findings: We mapped 8 *Ltr* (*Leishmania tropica* response) loci. Four loci (*Ltr2*, *Ltr3*, *Ltr6* and *Ltr8*) exhibit independent responses to *L. tropica*, while *Ltr1*, *Ltr4*, *Ltr5* and *Ltr7* were detected only in gene-gene interactions with other *Ltr* loci. *Ltr3* exhibits the recently discovered phenomenon of transgenerational parental effect on parasite numbers in spleen. The most precise mapping (4.07 Mb) was achieved for *Ltr1* (chr.2), which controls parasite numbers in lymph nodes. Five *Ltr* loci co-localize with loci controlling susceptibility to *L. major*, three are likely *L. tropica* specific. Individual *Ltr* loci affect different subsets of responses, exhibit organ specific effects and a separate control of parasite load and organ pathology.

Conclusion: We present the first identification of genetic loci controlling susceptibility to *L. tropica*. 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.

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Introduction

Leishmaniasis is endemic in 98 countries on 5 continents, causing 20,000 to 40,000 deaths per year [1]. In the past decade the number of endemic regions have expanded, prevalence has increased and the number of unrecorded cases must have been substantial, because notification has been compulsory in only 32 of the 98 countries where 350 million people are at risk [1,2]. Infection represents an important global health problem, as no safe and effective vaccine currently exists against any form of human leishmaniasis, and the treatment is hampered by serious side effects [3].

The disease is caused by obligate intracellular vector-borne parasites of the genus *Leishmania*. In the vertebrate host organism, *Leishmania* parasites infect so-called professional phagocytes (neutrophils, monocytes and macrophages) [4], as well as dendritic cells [5], immature myeloid precursor cells, sialoadhesin-positive stromal macrophages of the bone marrow, hepatocytes and fibroblasts [6]. Leishmaniasis includes asymptomatic infection and three main clinical syndromes. In the dermis, parasites cause the cutaneous form of the disease, which can be localized or diffuse; in the mucosa, they cause mucocutaneous leishmaniasis, and the metastatic spread of infection to the spleen and liver leads to visceral leishmaniasis (also known as kala-azar or black fever). Parasites can also enter other organs, such as lymph nodes, bone marrow and lungs, and in rare cases, can even reach the brain [4]. One of the major factors determining the type of pathology is

Author Summary

Leishmaniasis, a disease caused by *Leishmania* ssp. is among the most neglected infectious diseases. In humans, *L. tropica* causes cutaneous form of leishmaniasis, but can damage internal organs too. The reasons for this variability are not known, and its genetic basis was never investigated. Therefore, analysis of genes affecting host's responses to this infection can elucidate the characteristics of individual host-parasite interactions. Recombinant congenic strain CcS-16 carries 12.5% genes from the mouse strain STS/A on genetic background of the strain BALB/c, and it is more susceptible than BALB/c. 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 loci are functionally heterogeneous - each influences a different set of responses to the pathogen. Five loci co-localize with the previously described loci that control susceptibility to *L. major*, three are species-specific. *Ltr2* co-localizes not only with *Lmr14* (*Leishmania major* response 14), but also with *Ir2* influencing susceptibility to *L. donovani* and might therefore carry a common gene controlling susceptibility to leishmaniasis.

the species of *Leishmania* [7]. However, the transmitting vector, as well as genotype, nutritional status of the host, and environmental and social factors also have a large impact on the outcome of the disease [4,7]. That is why even patients infected by the same species of *Leishmania* develop different symptoms [7] and may differ in response to therapy [3]. The basis of this heterogeneity is not well understood [8], but part of this variation is likely genetic [4].

The search for loci and genes controlling leishmaniasis included candidate-gene approach, genome-wide linkage and association mapping. Genotyping of candidate genes, which have been chosen on the basis of previous immunological studies (hypothesis-driven approach) detected influence of polymorphism in *HLA-Cw7*, *HLA-DQw3*, *HLA-DR*, *TNFA* (tumor necrosis factor alpha), *TNFB*, *IL4*, *IFNGR1* (interferon gamma receptor 1) [reviewed in [4]], *TGFB1* (transforming growth factor, beta 1) [9], *IL1* [10], *IL6* [11], *CCL2/MCP1* (chemokine (C-C motif) ligand 2) [12], *CXCR1* (chemokine (C-X-C motif) receptor 1) [13], *CXCR2* (chemokine (C-X-C motif) receptor 2) [14], *FCN2* (ficolin-2) [15] and *MBL2* (mannose-binding lectin (protein C) 2) [16] on response to different human leishmaniases.

Hypothesis-independent search for susceptibility genes included genome-wide linkage and association mapping. Bucheton and coworkers [17] performed a genome-wide linkage scan, identified a major susceptibility locus that controls the susceptibility to *L. donovani* on chromosome 22q12 [17] and found that polymorphism in *IL2RB* (interleukin 2 receptor, beta chain) in this chromosomal region is associated with susceptibility to visceral leishmaniasis [18]. Genome-wide search with the subsequent analysis of a putative susceptibility locus on chromosome 6q27 revealed that polymorphism in *DLL1* (delta-like 1 (Drosophila)), the ligand for NOTCH3 (Neurogenic locus notch homolog protein 3) [19] is associated with susceptibility to visceral leishmaniasis caused by *L. donovani* and *L. infantum chagasi*. Delta1-Notch3 interactions bias the functional differentiation of activated $CD4^+$ T cells [20]. GWAS (genome-wide association study) established that common variants in the *HLA-DRB1-HLA-DQA1* HLA class II region contribute to susceptibility to *L. donovani* and *L. infantum chagasi* [21].

Genome-wide linkage in mouse revealed susceptibility genes *Nramp1* (Natural resistance-associated macrophage protein 1)/*Slc11a1* (solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1) [22] and *Fli1* (Friend leukaemia virus integration 1) [23] and the role of these genes has been also established in humans [13,24,25]. *NRAMP1*, which controls susceptibility to *L. donovani* and *L. infantum* functions as a divalent metal pH-dependent efflux pump at the phagosomal membrane of macrophages and neutrophils [26]. It is also expressed in dendritic cells and influences major histocompatibility complex class II expression and antigen-presenting cell function [27]. Susceptible mouse allele carries a "null" mutation that abolishes gene function (it is a natural knockout) [28], whereas polymorphisms in the promoter, exon3 and the intron of human *SLC11A1* [24], are expected to have a smaller impact on gene function. The Friend leukaemia virus integration gene, linked with wound healing, influences cutaneous leishmaniasis caused by *L. major* in mouse [23] and by *L. braziliensis* in human [25]. It remains to be tested, whether natural polymorphisms detected in mouse genes *bg* (beige)/*Lyst* (lysosomal trafficking regulator) [29] and cationic amino acid transporter *Slc7a2* (solute carrier family 7 (cationic amino acid transporter, y+ system), member 2) [30] influencing response to *L. donovani* [31] and *L. major* [30], respectively, plays role also in humans. However, nothing is known about genes controlling *L. tropica*-induced disease in humans.

L. tropica causes cutaneous leishmaniasis in humans, but it can also visceralize. Although cutaneous disease due to *L. tropica* is known for a long time, its potential to visceralize in humans has been recognized only relatively recently [32]. Visceralized *L. tropica* was also identified as the cause of an initially not understood systemic illness in veterans returning from endemic areas in the Middle East [33]. This finding stimulated interest in less typical symptoms induced by this parasite. It was found that *L. tropica* caused visceral disease in Kenya [34], as well as classical visceral leishmaniasis (kala-azar) in India [35,36] and in Iran [37], and disseminated cutaneous leishmaniasis accompanied with visceral leishmaniasis in Iran [38]. *L. tropica* was also implicated in development of mucosal leishmaniasis in Iran [39]. The reasons of this variability are not known.

A suitable animal model for study of this parasite would therefore contribute to genetic dissection of the functional and clinical manifestations of infection. Golden hamsters (*Mesocricetus auratus*) have been considered to be the best model host for *L. tropica* infection, but this host is not inbred and therefore not suitable for genetic dissection. Fortunately, several *L. tropica* strains from Afghanistan, India [40], and Turkey [41] have been reported to cause cutaneous disease in inbred BALB/c mice. Extension of analysis to the strains C57BL/6J, C57BL/10SgSnAi and gene-deficient mice on their backgrounds indicated role of IL-10 and TGFβ in regulation of parasite numbers in ears of infected mice [42].

We studied susceptibility to *L. tropica* using BALB/c-c-STS/A (CcS/Dem) recombinant congenic (RC) strains [43], which differ greatly in susceptibility to *L. major* [44,45]. Parental strains BALB/c, STS and RC strains CcS-3, CcS-5, CcS-11, CcS-12, CcS-16, CcS-18, and CcS-20 were infected with *L. tropica* and skin lesions, cytokine and chemokine levels in serum, splenomegaly, hepatomegaly, and parasite numbers in organs were measured [46]. These experiments revealed that manifestations of the disease after infection with *L. tropica* are strongly influenced by genotype of the host. We have found that females of the RC strain CcS-16 that

contains 12.5% genes of the resistant donor strain STS and 87.5% genes of the susceptible strain BALB/c [43,47] developed the largest skin lesions and exhibited a unique systemic chemokine reaction, characterized by additional transient early peaks of CCL3 and CCL5, which were present neither in CcS-16 males nor in any other tested RC strain [46]. In order to establish the genetic basis of these differences, we prepared F_2 hybrids between BALB/c and CcS-16, infected them with *L. tropica* and measured their skin lesions, splenomegaly, hepatomegaly, parasite numbers in spleen, liver and inguinal lymph nodes, and serum level of CCL3, CCL5 and CCL7 during the transient early peak. The strain CcS-16 carries STS-derived segments on nine chromosomes. They were genotyped in the F_2 hybrid mice and their linkage with pathological symptoms and systemic immune responses was determined, which revealed eight controlling genes.

Materials and Methods

Mice

Females of strains BALB/c (16 infected, 16 uninfected) and CcS-16 (15 infected, 11 uninfected) were 8 to 19 weeks old (mean age 12 weeks, median age 12 weeks) at the time of infection. When used for these experiments, strain CcS-16 was in more than 90 generations of inbreeding. The parts of its genome inherited from the BALB/c or STS parents were defined [48]. 247 female F_2 hybrids between CcS-16 and BALB/c (age 9 to 16 weeks at the time of infection, mean age 13 weeks, median 13 weeks) were produced at the Institute of Molecular Genetics AS CR, v.v.i.. Mice were kept in individually ventilated cages (Ehret, Emmendingen, Germany) and tested in two experimental groups. Both groups of F_2 hybrids were derived from the same F_1 parents; second experiment started seven weeks after the first. 2 mice died shortly after inoculation and were excluded from experiments. Among analyzed F_2 hybrids, first experiment consisted of 111 mice, of which 51 mice originated from a cross (BALB/c×CcS-16)F_2 (mean age 11.9 weeks, median 12 weeks; 3 mice died before the end of an experiment), 60 mice originated from a cross (CcS-16×BALB/c)F_2 (mean age 12.6 weeks, median age 13 weeks; 1 mouse died before the end of an experiment). According to the nomenclature rules, the first strain listed in the cross symbol is the female parent, the second the male. The second experiment contained 134 mice, of which 64 mice originated from a cross (BALB/c×CcS-16)F_2 (mean age 12.6 weeks, median 16 weeks; 2 mice died before the end of an experiment), 70 mice originated from a cross (CcS-16×BALB/c)F_2 (mean age 13.4 weeks, median age 13 weeks; 6 mice died before the end of an experiment). The numbers of mice analyzed for individual phenotypes are given in Supplementary Table S1.

Ethics statement

All experimental protocols utilized in this study comply with the Czech Government Requirements under the Policy of Animal Protection Law (No. 246/1992) and with the regulations of the Ministry of Agriculture of the Czech Republic (No. 207/2004), which are in agreement with all relevant European Union guidelines for work with animals and were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics AS CR and by Departmental Expert Committee for the Approval of Projects of Experiments on Animals of the Academy of Sciences of the Czech Republic (permission Nr. 37/2007).

Parasite

Leishmania tropica from Urfa, Turkey (MHOM/1999/TR/SU23) was used for infecting mice. Amastigotes were transformed to promastigotes using SNB-9 [49], and 1×10^7 stationary phase promastigotes from subculture 2 were inoculated in 50 µl of sterile Phosphate Buffer Saline (PBS) s.c. into the tail base, with promastigote secretory gel (PSG) collected from the midgut of *L. tropica*-infected *Phlebotomus sergenti* females (laboratory colony originating from *L. tropica* focus in Urfa). PSG was collected as described [50]. The amount corresponding to one sand fly female was used per mouse.

Disease phenotype

The size of the skin lesions was measured every second week using the Profi LCD Electronic Digital Caliper Messschieber Schieblehre Messer (Shenzhen Xtension Technology Co., Ltd. Guangdong, China), which has accuracy 0.02 mm. Blood was collected every 2 weeks in volume from 60 to 180 µl, and serum was frozen at −30°C for further analysis. The mice were killed 43 weeks after inoculation. Blood, spleen, liver and inguinal lymph nodes were collected for later analysis.

Quantification of parasite load

Parasite load was measured in frozen lymph nodes, spleen, and liver samples using PCR-ELISA according to the previously published protocol [51]. Briefly, total DNA was isolated using a TRI reagent (Molecular Research Center, Cincinnati, USA) standard procedure (http://www.mrcgene.com/tri.htm). For PCR, two primers (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) were used for amplification of the 120-bp conservative region of the kinetoplast minicircle of *Leishmania* parasite, and 50 ng of extracted DNA was used per each PCR reaction. For a positive control, 20 ng of *L. tropica* DNA per reaction was amplified as a highest concentration of standard. A 30-cycle PCR reaction was used for quantification of parasites in lymph nodes; 33 cycles for spleen, and 40 cycles for liver. Parasite load was determined by analysis of the PCR product by the modified ELISA protocol (Pharmingen, San Diego, USA). Concentration of *Leishmania* DNA was determined using the ELISA Reader Tecan and the curve fitter program KIM-E (Schoeller Pharma, Prague, Czech Republic) with least squares-based linear regression analysis.

Chemokines and cytokine 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 (eBioscience, Vienna, Austria). The kit contains two sets of beads of different size internally dyed with different intensities of fluorescent dye. The set of small beads was used for GM-CSF, CCL5/RANTES and CCL4/MIP-1β and the 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 a fluorescent signal. The test procedure was performed in the 96 well filter plates (Millipore, USA) according to the protocol of manufacturer. Beads were analyzed on flow cytometer LSR II (BD Biosciences, San Jose, USA). Lyophilized GM-CSF and chemokines (CCL2/MCP-1, CCL3/MIP1α, CCL4/MIP1β, CCL5/RANTES, CCL7/

Table 1. Loci that control skin lesion development.

Phenotype	Locus	Marker	Genotype						*P* value	corr. *P* value	Bonfer. corr. *P* value	% of explained variance
			CC		CS		SS					
lesion wk 19	*Ltr2*	**D2Nds3**	**0.00**	0.002±0.001 (n = 60)	**0.35**	0.004±0.001 (n = 127)	**1.65**	0.010±0.001 (n = 53)	6.6×10^{-5}	**0.004**	**0.049**	20.90
lesion wk 19	*Ltr4*	**D4Mit153**	**1.31**	0.008±0.001 (n = 54)	**0.23**	0.003±0.001 (n = 130)	**0.38**	0.004±0.001 (n = 56)	0.0018	0.077	0.93	9.66
lesion wk 21	*Ltr2*	**D2Nds3**	**0.20**	0.003±0.002 (n = 60)	**0.36**	0.004±0.001 (n = 127)	**2.27**	0.012±0.002 (n = 53)	2.9×10^{-5}	**0.002**	**0.024**	10.93
lesion wk 21	*Ltr3*	**D3Mit11**	**0.39**	0.004±0.002 (n = 64)	**0.42**	0.004±0.001 (n = 122)	**1.94**	0.011±0.002 (n = 54)	0.00088	**0.042**	0.50	12.48
lesion wk 23	*Ltr3*	**D3Mit11**	**0.25**	0.149±0.010 (n = 64)	**0.28**	0.152±0.008 (n = 122)	**1.29**	0.203±0.012 (n = 54)	0.00053	**0.026**	0.32	6.59
lesion wk 25	*Ltr3*	**D3Mit11**	**0.52**	0.069±0.008 (n = 64)	**0.48**	0.067±0.007 (n = 122)	**2.21**	0.110±0.009 (n = 54)	0.00048	**0.024**	0.29	12.02
lesion wk 27	*Ltr4*	**D4Mit153**	**1.75**	0.101±0.008 (n = 54)	**0.59**	0.071±0.006 (n = 130)	**0.30**	0.060±0.008 (n = 55)	0.00096	**0.045**	0.54	7.53
lesion wk 31	*Ltr2*	**D2Mit389**	**1.32**	0.092±0.012 (n = 55)	**1.57**	0.097±0.007 (n = 134)	**5.53**	0.148±0.011 (n = 48)	0.00028	**0.015**	0.18	10.77

Lesions were measured every second week. In order to normalize distribution of the observed values, the natural logarithm of the lesion size (mm^2) at each measured week (value+1.5) raised to the power of 0.04 was further raised to the power of 1.5 for weeks 19, 21, 23, 25; and to the power of 0.75 for week 31. The table shows means and SE calculated by analysis of variance. Non-transformed values of mean are given in bold. Number of tested mice is shown in brackets. Only *P* values significant after correction for genome-wide analysis and Bonferroni correction (multiplied by the number of tested weeks) are given. C and S indicate the presence of BALB/c and STS allele, respectively.

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MCP-3) supplied in the kit were used as standards. Concentration was evaluated by Flow Cytomix Pro 2.4 software (eBioscience, Vienna, Austria). 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, and for CCL5/RANTES 6.1 pg/ml.

Genotyping of F_2 mice

DNA was isolated from tails using a proteinase procedure [52] with modifications described in [51]. The strain CcS-16 differs from BALB/c at STS-derived regions on nine chromosomes [48 and unpublished results]. These differential regions were typed in the F_2 hybrid mice between CcS-16 and BALB/c using 23 microsatellite markers (Generi Biotech, Hradec Králové, Czech Republic): D2Mit156, D2Mit389, D2Nds3, D2Mit257, D2Mit283, D2Mit52, D3Mit25, D3Mit11, D4Mit153, D6Mit48, D6Mit320, D10Mit67, D10Mit103, D11Mit139, D11Mit242, D11Nds18, D11Mit37, D16Mit126, D17Mit38, D17Mit130, D18Mit35, D18Mit40 and D18Mit49 (Supplementary Table S2). The maximum distance between any two markers in the chromosomal segments derived from the strain STS or from the nearest BALB/c derived markers was 14.16 cM. The DNA genotyping by PCR was performed as described elsewhere [53]. The genotyping for microsatellite markers with fragment length difference of less than 10 bp was performed by using ORIGINS Elchrom Scientific electrophoresis (Elchrom Scientific AG, Cham, Switzerland) according to manufacturer's instruction. Briefly, DNA was amplified as described in [53]. Each PCR product was mixed with 5 µl of loading buffer and electrophoresed using Spreadex EL300 gel and Spreadex EL400 gel (Elchrom Scientific AG, Cham, Switzerland) for product with size of less than 150 bp or more than 150 bp, respectively. The best gel and proper running time was selected using ElQuantTM Software (Elchrom Scientific AG, Cham, Switzerland). 30 mM TAE buffer was used as a running buffer. Running temperature was set to 20°C and to 50°C, when voltage was set to 120 V and 100 V, respectively. After finishing the electrophoresis gel was stained by ethidium bromide and the results were read by GENE bio-imaging system (Syngene, Cambridge, UK).

Statistical analysis

The role of genetic factors in control of the tested pathological and immunological symptoms was examined with ANOVA using the program Statistica for Windows 8.0 (StatSoft, Inc., Tulsa, Oklahoma, USA). Marker, grandparent-of-origin effect and age were fixed factors and the experiment was considered as a random factor. In order to obtain normal distribution of the analyzed parameters, the obtained values were transformed, each as required by its distribution, as shown in the legends of the Tables. Markers and interactions with $P<0.05$ were combined in a single comparison.

To obtain whole genome significance values (corrected *P*-values) the observed *P*-values (αT) were adjusted according to Lander and Schork [54] using the formula:

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

where G = 1.75 Morgan (the length of the segregating part of the genome: 12.5% of 14 M); C = 9 (number of chromosomes segregating in cross between CcS-16 and BALB/c, respectively); ρ = 1.5 for F_2 hybrids; h(T) = the observed statistic (F ratio).

The percent of total phenotypic variance accounted for by a QTL and its interaction terms was computed by subtracting the

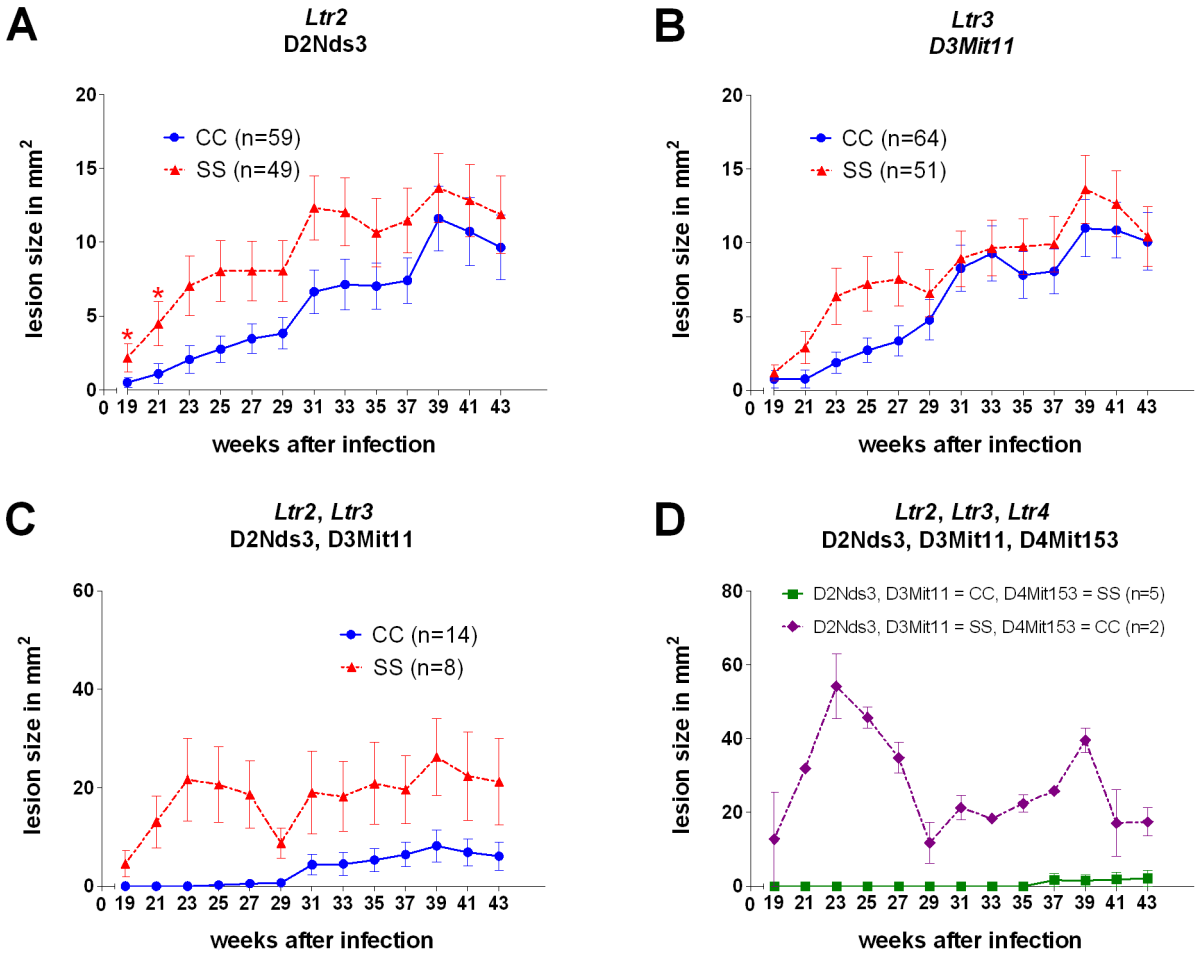

Figure 1. Differential lesion development in F_2 hybrid mice carrying one, two and three *Ltr* loci after infection with *L. tropica*. A. F_2 hybrids carrying BALB/c or STS homozygous (resistant or susceptible) alleles in *Ltr2* (D2Nds3); **B.** F_2 hybrids carrying BALB/c or STS homozygous (resistant or susceptible) alleles in *Ltr3* (D3Mit11); **C.** F_2 hybrids carrying BALB/c or STS homozygous (both resistant or both susceptible) alleles in both *Ltr2* (D2Nds3) and *Ltr3* (D3Mit11); **D.** F_2 hybrids carrying BALB/c homozygous (both resistant) alleles in *Ltr2* and *Ltr3* and STS (resistant) homozygous alleles in *Ltr4* (D4Mit153), and F_2 hybrids carrying STS homozygous (both susceptible) alleles in *Ltr2* and *Ltr3* and BALB/c (susceptible) homozygous alleles in *Ltr4*. n, number of mice. Graphs summarize data from two independent experimental groups and give non-normalized lesion sizes. Lesions were measured every second week. CC and SS indicate the homozygosity of BALB/c and STS allele, respectively. Please note different scales of Figures 1A,B, 1C and 1D.
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sums of squares of the model without this variable from the sum of squares of the full model and this difference divided by the total regression sums of squares:

$$\frac{(SS(b1,b2,b3,b4,b5|b0)) - (SS(b1,b2,b3,b4|b0))}{(RSS(b1,b2,b3,b4,b5|b0)}$$

Results

Genetic control of skin lesions development

Differences in skin lesions development between strains BALB/c and CcS-16 are controlled by two loci, which are not dependent on or influenced by interaction with other genes (main effects) (Table 1, Figure 1). *Ltr2* (*Leishmania tropica* response 2) linked to D2Nds3 (Figure 1A) and D2Mit389 influences lesion size at week 19 (corrected $P = 0.004$, Bonferroni corr. $P = 0.049$), 21 (corrected $P = 0.0020$, Bonferroni corr. $P = 0.024$) and 31 (corrected $P = 0.0152$, Bonferroni corr. $P = 0.18$) after infection, *Ltr3* that controls lesion size at week 21 after infection is linked to D3Mit11 (corrected $P = 0.042$, Bonferroni corr. $P = 0.5$) (Figure 1B). STS allele of both *Ltr2* and *Ltr3* determines larger lesions. STS allele of *Ltr4* marked by D4Mit153 (which also controls parasite numbers in liver and in lymph nodes) has an opposite effect on the studied trait; its STS allele is associated with smaller lesions at week 27 after infection. Figure 1C and Figure 1D show the strong additive effects of *Ltr2* and *Ltr3*, and *Ltr2*, *Ltr3* and *Ltr4*, respectively. However, *Ltr3* and *Ltr4* effects on skin lesions (nominal P value = 0.00048 and 0.00096, respectively, corr. P value = 0.024 and 0.045, respectively) were not significant after Bonferroni correction for number of tested weeks of infection and for whole genome significance. Although lesions were larger in the second experiment, no significant interaction between experimental group and markers was observed.

Genetic control of parasite numbers in organs and visceral pathology

Parasite numbers in spleen and splenomegaly are controlled by different sets of genes. Parasite numbers in

Table 2. Main effect loci: control of parasite load in spleen and in liver, and visceral pathology.

Phenotype	Locus	Marker	Genotype									*P* value	corr. *P* value	% of expl. variance
			CC			CS			SS					
Parasites in spleen														
Both crosses	*Ltr3*	**D3Mit25**	**0.80**	4.38±0.16	(n = 61)	**0.63**	4.15±0.13	(n = 108)	**0.48**	3.87±0.17	(n = 62)	0.094	NS	NA
(BALB/c×CcS-16)F_2	*Ltr3*	**D3Mit25**	**1.72**	5.15±0.22	(n = 29)	**0.75**	4.32±0.21	(n = 43)	**0.43**	3.76±0.22	(n = 37)	0.00014	**0.0085**	19.38
(CcS-16×BALB/c)F_2	*Ltr3*	**D3Mit25**	**0.38**	3.63±0.21	(n = 32)	**0.49**	3.89±0.15	(n = 65)	**0.61**	4.11±0.23	(n = 25)	0.304	NS	NA
Parasites in spleen														
Both crosses	*Ltr6*	**D11Mit37**	**0.57**	4.04±0.16	(n = 65)	**0.46**	3.84±0.12	(n = 105)	**0.96**	4.56±0.16	(n = 62)	0.0028	0.113	NA
(BALB/c×CcS-16)F_2	*Ltr6*	**D11Mit37**	**0.65**	4.17±0.24	(n = 31)	**0.45**	3.81±0.21	(n = 46)	**1.75**	5.17±0.23	(n = 32)	0.00024	**0.014**	29.58
Splenomegaly	*Ltr8*	**D18Mit49**	**5.28**	1.70±0.06	(n = 74)	**4.67**	1.57±0.05	(n = 106)	**3.60**	1.30±0.07	(n = 53)	0.00022	**0.012**	18.59
Parasites in liver	*Ltr2*	**D2Nds3**	**0.61**	4.12±0.11	(n = 60)	**0.83**	4.42±0.08	(n = 123)	**1.25**	4.83±0.14	(n = 49)	0.00056	**0.028**	9.50
Hepatomegaly	*Ltr2*	**D2Mit389**	**45.76**	37.41±0.81	(n = 55)	**42.28**	34.66±0.52	(n = 131)	**48.31**	39.42±0.86	(n = 46)	4.3×10^{-6}	**0.00033**	13.83

Parasite numbers (week 43) were estimated by PCR–ELISA. Means, SE and *P* values for splenomegaly (week 43), hepatomegaly (week 43) and concentration of parasite DNA (ng/μl) in isolates from lymph nodes, spleen and liver were calculated by analysis of variance. Normal distribution was obtained for splenomegaly (spleen-to-body weight ratio×1000) by raising values to the power of 0.00002. Hepatomegaly (liver-to-body weight ratio×1000) was normalized by raising values to the power of 0.0125. To obtain normal distribution for parasite load in organs, the following transformations were used: natural logarithm of (value×100). The numbers in bold give the average non-transformed values. Only *P* values significant after correction for genome-wide testing are given. Number of tested mice is shown in brackets. C and S indicate the presence of BALB/c and STS allele, respectively. NS – not significant, NA – not applicable.

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spleen are controlled by two loci (Table 2). STS allele of *Ltr3* linked with D3Mit25 (corrected $P = 0.0085$) determines lower parasite load, whereas STS allele of *Ltr6* (linked with D11Mit37) (corrected $P = 0.014$) is associated with higher parasite numbers. These *P*-values for *Ltr3* and *Ltr6* were significant only in cross (BALB/c×CcS-16)F_2 (where mother of the F_1 hybrids was BALB/c and father was CcS-16), but not in cross (CcS-16×BALB/c)F_2 (where mother was CcS-16 and father was BALB/c). Interaction between the cross and marker D3Mit25 is highly significant (corr. $P = 0.0013$). Younger mice (from 9 to 12 weeks, mean = 11 weeks) have higher parasite load than the older (from 13 to 16 weeks, mean = 14 weeks) mice, but interaction between the marker and age was not significant (nominal $P = 0.86$).

Splenomegaly is controlled by five loci (Table 2, 3). *Ltr8* linked with D18Mit49 (corr. $P = 0.012$) has a main effect, its BALB/c allele is associated with a larger spleen to body weight ratio. *Ltr2*, *Ltr3*, *Ltr5* and *Ltr7* affect splenomegaly in gene-gene interactions. *Ltr2* linked to D2Mit257 influences splenomegaly in interaction with *Ltr3* linked to D3Mit11 (corrected $P = 0.010$). F_2 mice with homozygous STS (SS) alleles at both *Ltr2* and *Ltr3* have the smallest splenomegaly. *Ltr5* linked to D10Mit103 influences splenomegaly in interaction with *Ltr8* linked to D18Mit49 (corrected $P = 0.029$). F_2 mice with homozygous STS (SS) alleles at both *Ltr5* and *Ltr8* have the smallest splenomegaly. *Ltr5* also influences splenomegaly in interaction with *Ltr7* linked to D17Mit30 (corrected $P = 0.029$). F_2 mice with homozygous BALB/c (CC) alleles at *Ltr5* and homozygous STS (SS) alleles at *Ltr7* have the most severe splenomegaly, the other genotypes show no pronounced differences.

Parasite numbers in liver are controlled by *Ltr2*, *Ltr4* and *Ltr8*, whereas hepatomegaly is influenced by *Ltr2* only. Parasite numbers in liver are controlled by three genes (Table 2, 4). *Ltr2* linked to D2Nds3 (corrected $P = 0.028$) has a main effect on parasite numbers in liver. Its STS allele is associated with a higher parasite load (Table 2). *Ltr4* linked to D4Mit153 influences parasite load in liver in interaction with *Ltr8* linked to D18Mit40 (corrected $P = 0.021$). F_2 mice with homozygous BALB/c (CC) alleles at *Ltr4* and heterozygous at *Ltr8* have the highest parasite burden in liver.

Hepatomegaly is determined by locus *Ltr2* linked to D2Mit389 (corrected $P = 0.00033$) (Table 2). Less severe hepatomegaly was observed in heterozygotes.

Genetic control of parasite load in inguinal lymph nodes. Parasite numbers in inguinal lymph nodes are influenced by interaction between *Ltr1* linked to D2Mit156 and *Ltr4* linked to D4Mit153 (corrected $P = 0.032$). Highest parasite load is observed in F_2 mice with homozygous STS (SS) alleles at *Ltr4* and homozygous BALB/c (CC) alleles at *Ltr1* (Table 4). There was no interaction between experimental group and markers (nominal $P = 0.89$).

Genetic control of early peak of chemokines level in serum of infected mice

Genetic analysis of F_2 hybrids has revealed identical genetic control of serum levels of CCL3 and CCL5 at week 7 after infection (Table 5, 6). *Ltr3* linked to D3Mit11 determines levels of both CCL3 (corrected $P = 0.0046$) and CCL5 (corrected $P = 0.010$), its BALB/c allele is associated with higher chemokine levels (Table 5). *Ltr3* has not only individual (main) effect on chemokines levels, but also influences levels of CCL3 (corrected $P = 0.014$) and CCL5 (corrected $P = 0.0012$) in interaction with *Ltr7* linked to D17Mit130. The largest effect is seen by *Ltr3* when *Ltr7* is SS. In that genotypic situation the *Ltr3* CC alleles cause more than 300×higher levels of CCL3 and 28×higher levels of CCL5 than the *Ltr3* SS alleles (Table 6). It is likely that this very large size of this effect in *Ltr7* SS mice makes the *Ltr3* effects visible as a main effect, although smaller, in F_2 hybrids irrespective of their *Ltr7* genotype.

CCL7 level is controlled with two loci with an opposite effect on the studied trait. The homozygosity for the STS allele of *Ltr2* (SS) determines higher CCL7 level (corrected $P = 0.002$), whereas

Table 3. Interaction between loci that control splenomegaly after 43 weeks of *L. tropica* infection.

				***P*= 0.00026**		**Corrected *P*= 0.010**		**% of explained variance = 9.05**
		D2Mit257 (*Ltr2*)						
		CC		**CS**		**SS**		
D3Mit11	CC	**3.98**	1.41±0.09	**4.58**	1.55±0.06	**5.24**	1.69±0.08	
(*Ltr3*)			(n = 14)		(n = 33)		(n = 17)	
	CS	**4.51**	1.54±0.06	**4.35**	1.50±0.04	**5.32**	1.71±0.08	
			(n = 36)		(n = 63)		(n = 19)	
	SS	**4.37**	1.50±0.10	**5.23**	1.69±0.06	**3.12**	1.16±0.13	
			(n = 13)		(n = 31)		(n = 7)	
				***P*= 0.00083**		**Corrected *P*= 0.029**		**% of explained variance = 6.78**
		D10Mit103 (*Ltr5*)						
		CC		**CS**		**SS**		
D18Mit49	CC	**5.03**	1.65±0.10	**5.03**	1.65±0.07	**5.81**	1.80±0.10	
(*Ltr8*)			(n = 17)		(n = 34)		(n = 23)	
	CS	**4.50**	1.53±0.08	**4.51**	1.54±0.07	**5.03**	1.65±0.07	
			(n = 28)		(n = 45)		(n = 33)	
	SS	**4.11**	1.44±0.12	**4.38**	1.51±0.08	**2.60**	0.97±.12	
			(n = 12)		(n = 32)		(n = 9)	
				***P*= 0.00083**		**Corrected *P*= 0.029**		**% of explained variance = 7.54**
		D10Mit67 (*Ltr5*)						
		CC		**CS**		**SS**		
D17Mit130	CC	**4.73**	1.59±0.10	**4.15**	1.45±0.07	**4.67**	1.57±0.09	
(*Ltr7*)			(n = 14)		(n = 32)		(n = 16)	
	CS	**4.36**	1.50±0.06	**5.06**	1.66±0.05	**4.29**	1.48±0.06	
			(n = 35)		(n = 62)		(n = 28)	
	SS	**6.11**	1.85±0.09	**4.07**	1.43±.09	**4.65**	1.57±0.10	
			(n = 16)		(n = 18)		(n = 12)	

Means, SE and *P* values for splenomegaly were calculated by analysis of variance. Normal distribution was obtained for splenomegaly (spleen-to-body weight ratio×1000) by raising values to the power of 0.00002. The numbers in bold give the average non-transformed values. Only *P* values significant after correction for genome-wide significance are given. Number of tested mice is shown in brackets. C and S indicate the presence of BALB/c and STS allele, respectively.

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Table 4. Interaction between loci controlling parasite burden in lymph nodes and liver 43 weeks after infection.

Interaction between loci that control parasite burden in liver							
				***P*= 0.00059**		**Corrected *P*= 0.021**	**% of explained variance = 8.11**
		D18Mit40 (*Ltr8*)					
		CC		**CS**		**SS**	
D4Mit153	CC	**0.85**	4.44±0.2	**1.19**	4.78±0.2	**0.51**	3.93±0.21
(*Ltr4*)			(n = 14)		(n = 25)		(n = 14)
	CS	**1.00**	4.61±0.14	**0.65**	4.17±0.09	**1.05**	4.65±0.18
			(n = 33)		(n = 72)		(n = 19)
	SS	**0.67**	4.21±0.2	**0.73**	4.28±0.18	**0.85**	4.44±0.20
			(n = 13)		(n = 22)		(n = 20)
Interaction between loci that control parasite burden in inguinal lymph nodes							
				***P*= 0.00094**		**Corrected *P*= 0.032**	**% of explained variance = 7.31**
		D4Mit153 (*Ltr4*)					
		CC		**CS**		**SS**	
D2Mit156	CC	**0.87**	4.46±0.32	**0.73**	4.29±0.25	**3.86**	5.96±0.45
(*Ltr1*)			(n = 17)		(n = 26)		(n = 8)
	CS	**0.74**	4.31±0.25	**0.67**	4.21±0.17	**0.50**	3.91±0.23
			(n = 26)		(n = 64)		(n = 31)
	SS	**1.78**	5.18±0.40	**0.71**	4.26±0.23	**0.34**	3.54±0.38
			(n = 10)		(n = 32)		(n = 12)

Means, SE and *P* values for concentration of parasite DNA (ng/μl) in isolates from lymph nodes and liver were computed by analysis of variance. The following transformations were used to obtain normal distribution: natural logarithm of (value×100). Hepatomegaly (liver-to-body weight ratio×1000) was normalized by raising values to the power of 0.0125. The numbers in bold give the average non-transformed values. Only *P* values significant after correction for genome-wide significance are given. Number of tested mice is shown in brackets. C and S indicate the presence of BALB/c and STS allele, respectively.

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Table 5. Main effect of loci controlling serum chemokine level after 7 weeks of infection.

Phenotype	Locus	Marker	Genotype									*P* value	corr. *P* value	% of explained variance
			CC			CS			SS					
CCL3	*Ltr3*	**D3Mit11**	**711.42**	3.72±0.18	(n = 64)	**371.57**	3.27±0.12	(n = 118)	**94.68**	2.49±0.21	(n = 53)	7.5×10^{-5}	**0.0046**	4.56
CCL5	*Ltr3*	**D3Mit11**	**2724.44**	5.15±0.08	(n = 64)	**1805.94**	4.98±0.05	(n = 117)	**861.34**	4.66±0.09	(n = 53)	0.00018	**0.010**	3.99
CCL7	*Ltr2*	**D2M52**	**566.41**	6.34±0.05	(n = 48)	**590.60**	6.38±0.03	(n = 127)	**740.99**	6.61±0.05	(n = 60)	3×10^{-5}	**0.002**	9.06
CCL7	*Ltr8*	**D18M40**	**766.86**	6.64±0.05	(n = 60)	**602.67**	6.40±0.04	(n = 118)	**613.11**	6.42±0.06	(n = 55)	0.00024	**0.013**	11.38

In order to normalize distribution of the observed values (in pg/ml), the following transformations were used: the power of 0.2 (concentration value+1) – CCL3/MIP1α; natural logarithm – CCL7/MCP-3; the power of −0.117545 followed by subtraction with 1 – CCL5/RANTES. In case of CCL5/RANTES, the calculated value was further divided by −0.117545. The Table gives mean of non-transformed (in bold) and transformed concentration and SE of the transformed values calculated by analysis of variance. Only *P* values significant after correction for genome-wide significance are given. Number of tested mice is shown in brackets. C and S indicate the presence of BALB/c and STS allele, respectively.

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homozygosity for the BALB/c allele of *Ltr8* (CC) is associated with higher level of this chemokine (corrected $P = 0.013$) (Table 5). No significant interaction between experimental group and marker was observed. Older mice had higher levels of CCL7 in serum than the younger ones, but we did not observe any interactions between marker and age (nominal P (*Ltr2*) = 0.80, nominal P (*Ltr8*) = 0.64). Levels of CCL7 in serum of infected mice are also influenced by interaction of *Ltr2* linked to D2Mit257 and *Ltr6* linked to D11Mit37 (corrected $P = 0.016$), the highest CCL7 levels are observed in STS allele (SS) homozygotes in *Ltr6* in combination with heterozygotes (CS) or STS allele (SS) homozygotes in *Ltr2* (Table 6).

Although chemokine levels were higher in the first experiment, no significant interaction between experimental group and markers was observed.

No linkage was found for GM-CSF, CCL2/MCP-1 and CCL4/MIP-1β.

Discussion

The present study provides the first insight into the genetic architecture of susceptibility to *L. tropica*. We have described eight loci on seven chromosomes (Figure 2 [10,12,55–83]) and shown that the presence of individual symptoms of disease is controlled by different subsets of host's genes. The identification of host's genes responsible for the specific symptoms of the disease induced by different *Leishmania* species will contribute to the understanding of mechanisms of pathogenesis of leishmaniasis, similarly as comparative parasite genomics led to identification of differentially distributed genes in *Leishmania* species inducing different pathology [84,85], and analysis of specific virulence factors revealed how different *Leishmania* species subvert or circumvent host's defenses [7]. Such analysis will provide description of individual predisposition to specific symptoms of disease and its probable course. Moreover, the possibility to compare genetics of response to several *Leishmania* species will further help to understand the genetic basis of general and species-specific responses of the host. This will synergize with the future information about genome sequence of *L. tropica* and about interaction of its specific virulence factors with the immune system.

Response to *L. tropica* is controlled by multiple genes with heterogeneous effects

Our data show that interaction of mice with *L. tropica* parasites is complex and involves numerous genes and responses (Table 7). We have detected eight loci that in the strain CcS-16 control host-parasite interaction (Table 7, Figure 2). All eight *Ltr* loci are involved in gene-gene interactions (Figure 3), four loci (*Ltr2*, *Ltr3*, *Ltr6*, *Ltr8*) have also individual effect, while effects of *Ltr1*, *Ltr4*, *Ltr5* and *Ltr7* are seen only in interaction with other *Ltr* loci. This is not surprising, as the average proportion of genetic variation explained by epistatic QTLs in mice in different systems was estimated to be 49% [86] and gene-gene interactions were observed also in response to other pathogens such as *L. major* [87–89], *Trypanosoma brucei brucei* [53], *Salmonella enteritidis* [90], *Plasmodium falciparum* [91] and *Mycobacterium leprae* [92].

The loci described here have heterogeneous effects (Table 7). *Ltr1* on chromosome 2 controls in interaction with *Ltr4* only parasite numbers in lymph nodes, whereas the more distal *Ltr2* on the same chromosome influences development of skin lesions, splenomegaly (in interaction with *Ltr3*), hepatomegaly, parasite load in liver and level of CCL7 in serum. Multiple functions are also exerted by *Ltr3* on chromosome 3, which controls splenomegaly (in interaction with *Ltr2*), parasite numbers in spleen, and levels of CCL3 and CCL5 in serum. We have analyzed genetic control of early levels of chemokines, as there is a unique early peak in the CcS-16 females [46]. However, comparison of genetic control of CCL3 and CCL5 levels with genetic control of development of skin lesions indicates that there is no simple correlation between the chemokines levels and manifestations of disease. *Ltr4* on chromosome 4 controls in interaction with *Ltr1* and *Ltr8* parasite numbers in lymph nodes and in liver, respectively. *Ltr5* on chromosome 10 influences in interaction with *Ltr7* or *Ltr8* splenomegaly. *Ltr6* influences parasite numbers in spleens and level of CCL7 in serum (in interaction with *Ltr2*). *Ltr7* controls splenomegaly (in interaction with *Ltr5*) and in interaction with *Ltr3* level of both CCL3 and CCL5 in serum. *Ltr8* controls splenomegaly (as a main effect gene and in interaction with *Ltr5*), parasite numbers in liver (in interaction with *Ltr4*) and level of CCL7 in serum. *Ltr1* and *Ltr5* control only one parameter, whereas other loci have multiple effects. Some multiple effects could reflect causal relationship – e.g. CCL7 influences recruitment of monocytes to spleen [93], which could contribute to splenomegaly. The observed multiple effects of some *Ltr* loci might also suggest that some such loci might represent complexes of two or more closely linked *Ltr* genes. This issue will be resolved by future recombinational analysis.

We have detected also loci that control symptoms, such as splenomegaly, in which the strains BALB/c and CcS-16 do not differ [46]. This is because in an inbred strain the final outcome of

Table 6. Interaction between loci that control chemokines level after 7 weeks of *L. tropica* infection.

A. CCL3/MIP-1α				***P*=0.00036**		**Corrected *P*=0.014**		**% of variance = 3.33**
		D3Mit11 (*Ltr3*)						
		CC		**CS**		**SS**		
D17Mit130 (*Ltr7*)	CC	**298.60**	3.13±0.36	**377.81**	3.28±0.22	**172.79**	2.81±0.30	
			(n = 12)		(n = 32)		(n = 17)	
	CS	**358.96**	3.25±0.20	**248.17**	3.02±0.17	**288.44**	3.11±0.23	
			(n = 40)		(n = 58)		(n = 30)	
	SS	**2511.51**	4.79±0.36	**531.34**	3.51±0.24	**8.15**	1.56±0.51	
			(n = 12)		(n = 28)		(n = 6)	
B. CCL5/RANTES				***P*=2.7×10^{-5}**		**Corrected *P*=0.0012**		**% of variance = 3.96**
		D3Mit11 (*Ltr3*)						
		CC		**CS**		**SS**		
D17Mit130 (*Ltr7*)	CC	**1730.31**	4.97±0.15	**2023.41**	5.03±0.10	**2298.59**	5.08±0.14	
			(n = 12)		(n = 32)		(n = 17)	
	CS	**1977.17**	5.02±0.08	**1616.45**	4.94±0.07	**2338.11**	5.09±0.11	
			(n = 40)		(n = 57)		(n = 30)	
	SS	**7542.52**	5.53±0.15	**2144.55**	5.05±0.10	**272.74**	4.11±0.22	
			(n = 12)		(n = 28)		(n = 6)	
C. CCL7/MCP-3				***P*=0.00044**		**Corrected *P*=0.016**		**% of variance = 6.66**
		D2Mit257 (*Ltr2*)						
		CC		**CS**		**SS**		
D11Mit37 (*Ltr6*)	CC	**579.33**	6.36±0.08	**573.80**	6.35±0.05	**547.09**	6.30±0.10	
			(n = 17)		(n = 36)		(n = 10)	
	CS	**693.53**	6.54±0.07	**585.28**	6.37±0.04	**683.23**	6.53±0.07	
			(n = 30)		(n = 58)		(n = 22)	
	SS	**513.90**	6.24±0.08	**736.45**	6.60±0.06	**725.32**	6.59±0.09	
			(n = 17)		(n = 32)		(n = 13)	

In order to normalize distribution of the observed values, the concentration in pg/ml was raised to the power of 0.2 (concentration value+1) – CCL3/MIP1α; to the power of −0.117545 followed by subtraction with 1 – CCL5/RANTES. In case of CCL5/RANTES, the calculated value was further divided by −0.117545. The Table gives mean of non-transformed (in bold) and transformed concentration and SE of the transformed values calculated by analysis of variance. Only *P* values significant after correction for genome-wide significance are given. Number of tested mice is shown in brackets. C and S indicate the presence of BALB/c and STS allele, respectively.

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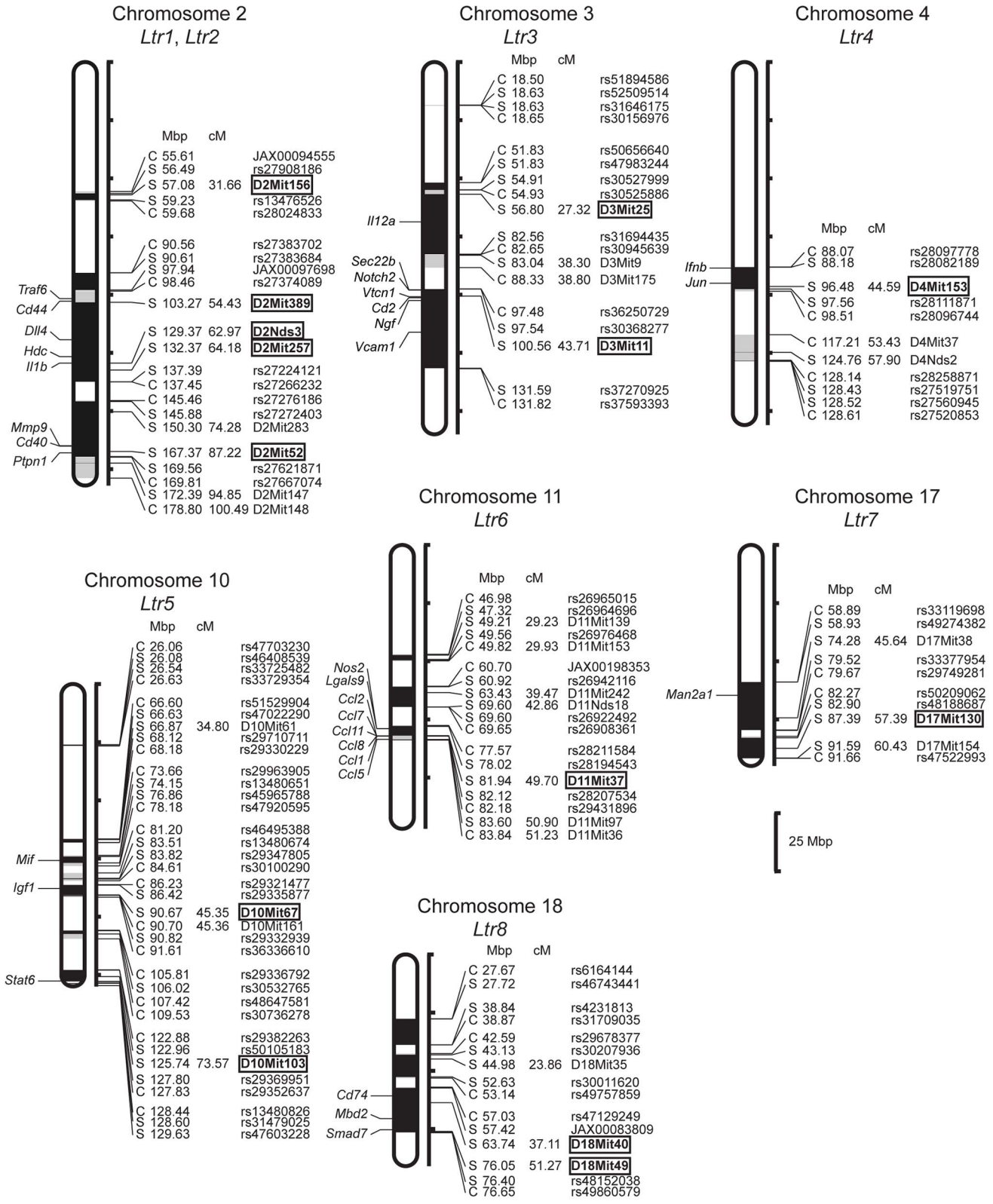

Figure 2. Position of the loci that control response to *L. tropica* in strain CcS-16. The regions of STS and BALB/c origin are represented as dark and white, respectively; the boundary regions of undetermined origin are shaded. Only the markers and SNPs defining the boundaries the STS-derived segment 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 bold. Abbreviations show genes that have been reported to be involved in response to *Leishmania* ssp.: *Ccl1* (chemokine (C-C motif) ligand 1) [55], *Ccl11* (chemokine (C-C motif) ligand 11) [56], *Ccl2* (chemokine (C-C motif) ligand 2), *Ccl5* (chemokine (C-C motif) ligand 5) [57], *Ccl7* [58], *Cd2* (CD2 antigen) [59], *Cd40* (CD40 antigen) [60], *Cd44* (CD44 antigen) [61], *Cd74* (CD74 antigen) [62], *Dll4* (Delta-like 4) [63], *Hdc* (histidine decarboxylase) [64], *Ifnb1* (interferon beta 1) [65], *Igf1* (insulin-like growth factor 1) [66], *Il1* (interleukin 1) [67], *Il12a* (Interleukin 12a) [68], *Jun* (Jun

oncogene) [69], *Lgals9* (lectin, galactose binding, soluble 9) [70], *Man2a1* (mannosidase 2, alpha 1) [71], *Mbd2* (methyl-CpG binding domain protein 2) [72], *Mif* (macrophage inhibitory factor) [73], *Mmp9* (matrix metalopeptidase 9) [74], *Ngf* (nerve growth factor) [75], *Nos2* (nitric oxide synthase 2, inducible) [76], *Notch2* (notch 2) [77], *Ptpn1* (protein tyrosine phosphatase, non-receptor type 1) [78], *Sec22b* (SEC22 vesicle trafficking protein homolog B (*S. cerevisiae*)) [79], *Smad7* (SMAD family member 7) [80], *Stat6* (Signal transducer and activator of transcription-6) [81], *Traf6* (TNF receptor associated factor 6) [60], *Vcam1* (vascular cell adhesion molecule 1) [82], *Vtcn1* (V-set domain containing T cell activation inhibitor 1) [83]. (Genes IDs are shown in Supplementary Table S3).
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response is exerted by multiple genes, which often have opposite effects, masking each other. In the F_2 hybrids these genes segregate and can be therefore detected.

Reliability and validity of the described loci is supported by the fact that they have been detected by analysis of different phenotypes and their statistical significance was corrected for whole genome testing and where appropriate also by conservative Bonferroni correction. The relatively high proportion of variance explained by the mapped loci (Table 1–6) might be partly due to a limited variability of the tested manifestations of the disease.

Susceptibility alleles carried by a resistant strain

Most inbred mouse strains that were produced without intentionally selectively bred for a specific quantitative phenotype (like susceptibility to specific infections) inherited from their non-inbred ancestors randomly susceptible alleles at some loci and resistant alleles at others, so that their overall susceptibility phenotype depends on the relative number of both. STS is resistant to *L. tropica* and does not develop skin lesions [24], however some STS-derived segments carried by CcS-16 on chromosome 2 (*Ltr2*) and possibly also on chromosome 3 (*Ltr3*) are associated with larger lesions. Similarly, STS-derived alleles of *Ltr2* and *Ltr6* are associated with higher parasite load in liver and spleen, respectively. This finding is not unique as susceptibility alleles originating from resistant strains were found in studies of colon cancer [94] and *L. major* [95] susceptibility; a low-responder allele was identified in a strain exhibiting high response to IL-2 [96] or producing a high level of IFNγ [97], whereas a high responder allele was found in a strain producing low level of IL-4 [98].

Transgenerational parental effect

Loci *Ltr3* and *Ltr6* influencing parasite numbers in spleen (Table 2) were significant only in the cross (BALB/c×CcS-16)F_2, but not in the cross (CcS-16×BALB/c)F_2, hence the outcome in these crosses that are theoretically genetically identical depends on the strain of the female or male used originally to produce the F_1 hybrids, which were then crossed with each other to produce the F_2 hybrids for the tests. Thus, this is a special type of a transgenerational parental effect as the mothers and fathers of the F_2 hybrids were genetically identical. Recently, examples of transgenerational parental effects have been described in several species [reviewed in [99]] and several possible mechanisms have been proposed. Our observation may reflect a parental effect due to modification of the developing immune system of fetuses or youngs by maternal environment, maternal nutritional effects, or epigenetic effects, and it offers a possibility to characterize the transgenerationally regulated functional pathways.

Control of parasite load is predominantly organ specific

Control of parasite elimination differs among organs: the loci *Ltr1* and *Ltr4* interact to control parasite numbers in inguinal lymph nodes, while *Ltr4* in interaction with *Ltr8* influences parasite load in liver (Table 4). Parasite load in liver is also controlled by *Ltr2* (Table 2), whereas parasite burden in spleen is influenced by *Ltr3* and *Ltr6* (Table 2). These data show that parasite elimination in lymph nodes, liver and spleen are controlled differently, suggesting a predominantly organ specific control of parasite load. Mechanistic studies analyzing response to *L. tropica* in different organs are not yet available, but generally organ specific responses described here are compatible with the mechanistic studies of other parasites. 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 [76,100]. Inducible nitric oxide synthase has been shown to control resistance to parasites in skin and draining lymph nodes, but not in spleen of the resistant strain C57BL/6 [100]. 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 [76]. Similarly, *bg/Lyst* (lysosomal trafficking regulator) is involved in control of parasite numbers of *L. donovani* in spleen, but not in liver [31]. On the

Table 7. Summary of loci that control response to *L. tropica*.

chr.	locus	marker	Phenotype controlled
2	*Ltr1*	D2Mit156	parasites in lymph nodes (int. *Ltr4* - D4Mit153)
2	*Ltr2*	D2Mit389; D2Nds3/*Il1b*; D2Mit257; D2Mit52	skin lesions wk 19; skin lesions wk 21; splenomegaly (int. *Ltr3* - D3Mit11); parasites in liver; hepatomegaly; CCL7; CCL7 (int. *Ltr6* - D11Mit37)
3	*Ltr3*	D3Mit25; D3Mit11	splenomegaly (int. *Ltr2* - D2Mit257); parasites in spleen (transgenerational parental effect); CCL3; CCL3 (int. *Ltr7* - D17Mit130); CCL5; CCL5 (int. *Ltr7* - D17Mit130)
4	*Ltr4*	D4Mit153	parasites in lymph nodes (int. *Ltr1* - D2Mit156); parasites in liver (int. *Ltr8* - D18Mit40)
10	*Ltr5*	D10Mit67; D10Mit103	splenomegaly (int. *Ltr7* - D17Mit130); splenomegaly (int. *Ltr8* - D18Mit49)
11	*Ltr6*	D11Mit37	parasites in spleen (transgenerational parental effect); CCL7 (int. *Ltr2* - D2Mit257)
17	*Ltr7*	D17Mit130	splenomegaly (int. *Ltr5* - D10Mit67); CCL3 (int. *Ltr3* - D3Mit11); CCL5 (int. *Ltr3* - D3Mit11)
18	*Ltr8*	D18Mit40; D18Mit49	splenomegaly; splenomegaly (int. *Ltr5* - D10Mit103); parasites in liver (int. *Ltr4* - D4Mit153); CCL7

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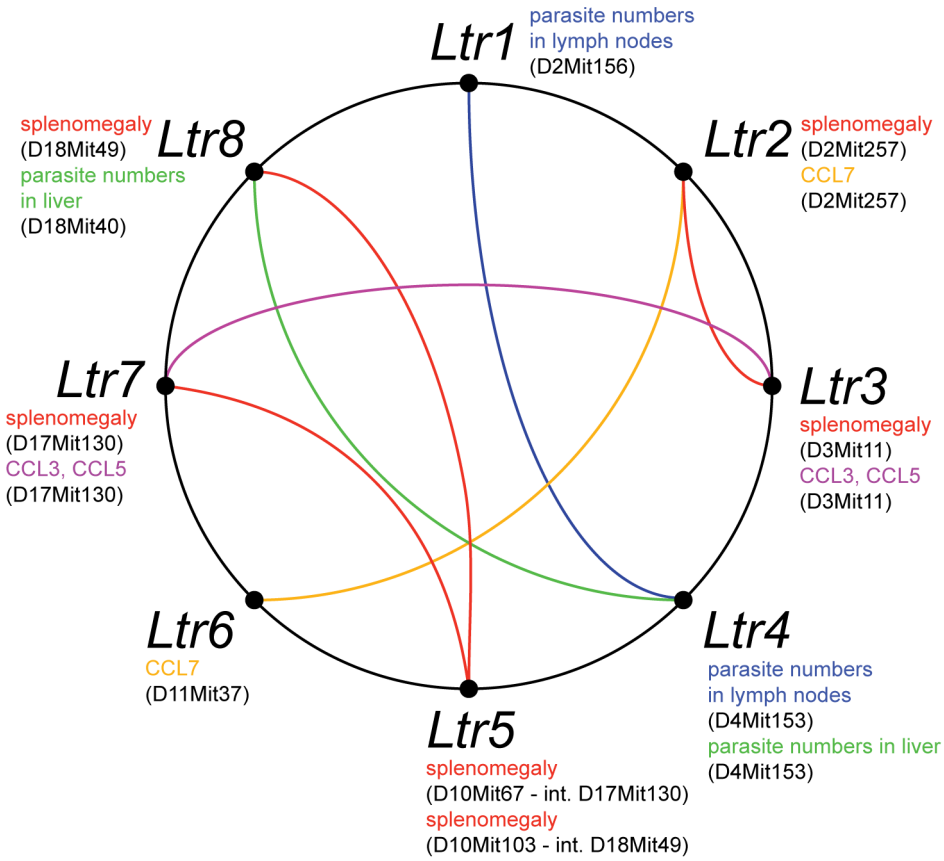

Figure 3. Interactions among loci that control response to *L. tropica*. Phenotypes controlled by each locus are shown at its symbol in different colors. The colored lines connecting the loci indicate interactions controlling the specific phenotypes.
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other hand VCAM-1 (vascular cell adhesion molecule-1) and VLA-4 (very late antigen-4) interactions influenced early *L. donovani* burden in liver, but not in spleen [82].

Different control of parasite elimination and organ pathology

Comparison of genetic control of parasite numbers in spleen and splenomegaly, or parasite numbers in liver and hepatomegaly shows that control of parasites elimination and organ pathology overlap only partially. For example *Ltr3* controls both parasite numbers in spleen and splenomegaly, but *Ltr6* is involved in control of parasite numbers in spleen, but not in splenomegaly, whereas *Ltr2*, *Ltr8*, *Ltr5*, and *Ltr7* are involved only in control of splenomegaly (Table 2, 3, 7). Similarly, *Ltr2* influences both parasite load in liver and hepatomegaly, but parasite load in liver is controlled also by interaction of *Ltr4* with *Ltr8*. The differences in genetic control of parasite numbers and organ pathology induced by the parasites are probably due to the fact that during a chronic disease the organ damage is a combined result of speed of elimination of parasite on one hand and changes caused by reaction to parasite (such as influx of immune cells, inflammatory responses) and healing processes on the other hand. It is therefore likely that these processes are regulated by different sets of genes.

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.

Comparison of genetic control of response to several pathogens

Comparison of loci that control response to *L. tropica* and *L. major* – indication of common and species-specific genes. Comparison of genetic control of response to *L. tropica* and *L. major* might indicate some common and some distinct mechanisms in response to these two parasites. We compared genetic relationship between the *Ltr* (this study) and *Lmr* [88,95, 101] loci detected in the strain CcS-16. Loci *Ltr1* (chromosome 2), *Ltr4* (chromosome 4) and *Ltr7* (chromosome 17) appear to be species-specific and do not overlap with loci controlling response to *L. major*. *Ltr2* (chromosome 2) co-localizes with *Lmr14*, *Ltr5* (chromosome 10) with *Lmr5*, *Ltr6* (chromosome 11) with *Lmr15*, and *Ltr8* (chromosome 18) with *Lmr13*. *Ltr2* controls visceral pathology in both species and is also involved in additional responses, which are unique for each parasite. Moreover, *Ltr2* and *Lmr14* overlap with *Ir2*, which controls visceral pathology after infection with *L. donovani* [4]. The other co-localizing loci also influence different sets of symptoms and are often involved in different interactions. This might indicate either the presence of the same controlling genes, which function differently under exposure to *L. tropica* and *L. major*, or less likely, a chance

coincidence – presence of different controlling genes on the same chromosomal segment.

Ltr3 on chromosome 3 co-localizes with *Lmr11*, which was detected in the strain CcS-20, but not in the CcS-16, and which exhibits a single gene effect on IL-6 level in serum [88] and in interaction with *Lmr8* on chromosome 1 influences serum IgE level in *L. major*-infected mice [101].

Some loci affect susceptibility to several pathogens. Some loci affect responses to a very broad spectrum of pathogens. For example, locus *Ltr2* co-localizes also with *Bb15*, which controls specific and total IgG in serum after infection with *Borrelia burgdorferi* [102]. The most obvious potential candidate gene in this chromosomal segment is *Il1* (interleukin 1). IL-1β was found to be up-regulated in dermal lesions of patients with cutaneous leishmaniasis caused by *L. tropica* and decreased after therapy [103], IL-1 was also found to regulate visceral manifestation of murine leishmaniasis after infection with *L. major* [67], and polymorphism in *IL1B* was linked with disease severity in patients infected with *L. mexicana* [10]. IL-1 was also described to influence IgG level in autoimmunity [104], which might suggest its involvement in response to *B. burgdorferi*.

Potential candidate genes

Usually, a standard inbred-strain mapping experiment using F_2 hybrids will map a QTL into a 20- to 40-cM interval [105]. In the RC strains 54% of their donor strain genome reside in segments of medium length (5–25 cM) [106]. However, RC strains can carry on some chromosomes very short segments of the donor strain origin. This feature of the RCS system allowed us previously to narrow the location of *Lmr9* (*Leishmania major* response 9) on chromosome 4 to a segment of 1.9 cM (6.79 Mb) without any additional crosses [101]. The short length of this segment, which controls levels of serum IgE in *L. major* infected mice, enabled us to detect a human homolog of this locus on human chromosome 8q12 and show that it controls susceptibility to atopy [107]. In another study, we were able to precisely map *Tbbr2* (*Trypanosoma brucei brucei* response 2) to 2.15 Mb [53].

In the present F_2 mapping experiment the shortest locus *Ltr1* is 4.07 Mb long (Figure 2). Although most *Ltr* loci contain several possible candidate genes, here we list (Figure 2)[10,12,55–83] only those that have been shown previously to influence infection with *Leishmania* ssp.. However, the effects of many of *Ltr* loci might be caused by genes that are at the present not considered as candidates. Currently we are producing mice with recombinant haplotypes that carry individual *Ltr* loci 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.

Conclusion

We present the first description of genetic architecture of response to *L. tropica* in any species. We observed organ specific control of infection and distinct control of parasite load and organ pathology, the typical characteristics of immune response to many pathogens observed in all infections where multiple disease parameters were studied (*L. major* [4], *L. donovani* [4], *Borrelia burgdorferi* [102], *Toxoplasma gondii* [108], *Trypanosoma congolense* [109], and *Chlamydia psittaci* [110]). In addition, the genetic control of response to *L. tropica* exhibits heterogeneity of gene effects, gene-gene interactions, and trans-generational parental effects. These complexities of genetic control have been invoked [111] to explain the very large fraction of heritability that has not been detectable in genome-wide association studies (GWAS) [112], a power deficiency that likely cannot be ameliorated by further increases of the number of tested SNPs or by whole genome sequencing. Identification of these complexities in the present study will open way to elucidation of their functional basis and detection of homologous processes in humans.

Supporting Information

Table S1 Numbers of mice analyzed in individual phenotypes.
(XLS)

Table S2 Chromosomal positions of typed markers.
(XLS)

Table S3 ID numbers of potential candidate genes localized in *Ltr* loci.
(XLS)

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Author Contributions

Conceived and designed the experiments: YS HH TK ML. Performed the experiments: YS HH TK MŠ VV IG TJ IK JV MS. Analyzed the data: YS HH TK VV PD ML. Wrote the paper: YS TK PD ML.

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4.3 Gene-specific sex effects on eosinophil infiltration in leishmaniasis

We tested eosinophil infiltration after *L. major* infection in parental strains BALB/cHeA and STS and RCS: CcS-1, CcS-3, CcS-4, CcS-5, CcS-7, CcS-9, CcS-11, CcS-12, CcS-15, CcS-16, CcS-18 and CcS-20. We sacrificed tested mice 8 weeks after inoculation and collected inguinal lymph nodes draining the site of infection for further analysis. We prepared haematoxylin stained tissue sections, examined them under a light microscope and assessed eosinophil infiltration using a semi-quantitative scoring system. Unexpectedly, eosinophil infiltration in strain CcS-9 exceeded that in parental BALB/cHeA and STS and was higher in males than in females. In the next step, we performed an analogous experiment with 254 F_2 hybrids between CcS-9 and BALB/cHeA in order to map genetic control of eosinophil infiltration in leishmaniasis. In the end of the experiment, we also collected tails for DNA isolation. We genotyped F_2 hybrids using 18 microsatellite markers covering STS regions of strain CcS-9. We also determined eosinophils infiltration into inguinal lymph nodes as described above and measured parasite load in lymph nodes using PCR-ELISA [147]. Using statistical analysis, we were able to find four novel loci controlling eosinophil infiltration after *L. major* infection named *Lmr14*, *Lmr15*, *Lmr25* and *Lmr26*. *Lmr14* (chr. 2) and *Lmr25* (chr. 5) operate independently from other genes (main effects). *Lmr14* functions only in males, the effect of *Lmr25* is sex independent. *Lmr15* (chr. 11) and *Lmr26* (chr. 9) operate in cooperation (non-additive interaction) with each other. This interaction was significant in males only, but sex-marker interaction was not significant. Eosinophil infiltration was positively correlated with parasite load in lymph nodes of F_2 hybrids in males, but not in females.

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Gene-specific sex effects on eosinophil infiltration in leishmaniasis

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Abstract

Background: Sex influences susceptibility to many infectious diseases, including some manifestations of leishmaniasis. The disease is caused by parasites that enter to the skin and can spread to the lymph nodes, spleen, liver, bone marrow, and sometimes lungs. Parasites induce host defenses including cell infiltration, leading to protective or ineffective inflammation. These responses are often influenced by host genotype and sex. We analyzed the role of sex in the impact of specific gene loci on eosinophil infiltration and its functional relevance.

Methods: We studied the genetic control of infiltration of eosinophils into the inguinal lymph nodes after 8 weeks of *Leishmania major* infection using mouse strains BALB/c, STS, and recombinant congenic strains CcS-1,-3,-4,-5,-7,-9,-11,-12, -15,-16,-18, and -20, each of which contains a different random set of 12.5% genes from the parental "donor" strain STS and 87.5% genes from the "background" strain BALB/c. Numbers of eosinophils were counted in hematoxylin-eosin-stained sections of the inguinal lymph nodes under a light microscope. Parasite load was determined using PCR-ELISA.

Results: The lymph nodes of resistant STS and susceptible BALB/c mice contained very low and intermediate numbers of eosinophils, respectively. Unexpectedly, eosinophil infiltration in strain CcS-9 exceeded that in BALB/c and STS and was higher in males than in females. We searched for genes controlling high eosinophil infiltration in CcS-9 mice by linkage analysis in F_2 hybrids between BALB/c and CcS-9 and detected four loci controlling eosinophil numbers. *Lmr14* (chromosome 2) and *Lmr25* (chromosome 5) operate independently from other genes (main effects). *Lmr14* functions only in males, the effect of *Lmr25* is sex independent. *Lmr15* (chromosome 11) and *Lmr26* (chromosome 9) operate in cooperation (non-additive interaction) with each other. This interaction was significant in males only, but sex-marker interaction was not significant. Eosinophil infiltration was positively correlated with parasite load in lymph nodes of F_2 hybrids in males, but not in females.

Conclusions: We demonstrated a strong influence of sex on numbers of eosinophils in the lymph nodes after *L. major* infection and present the first identification of sex-dependent autosomal loci controlling eosinophilic infiltration. The positive correlation between eosinophil infiltration and parasite load in males suggests that this sex-dependent eosinophilic infiltration reflects ineffective inflammation.

Keywords: *Leishmania major*, Mouse model, Eosinophil infiltration, Genetic control, QTL, Sex influence

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Background

Sex influences susceptibility to many infectious diseases [1], including some manifestations of leishmaniasis [2], a disease that threatens several hundred million people in 98 countries [3]. Disability-adjusted life years (DALYs) due to leishmaniasis are globally increasing [4]. The disease is caused by intracellular protozoan parasites of the genus *Leishmania* and is transmitted to the vertebrates by the bite of female phlebotomine sand flies.

Leishmania parasites infect the so-called professional phagocytes (neutrophils, monocytes, and macrophages), as well as dendritic cells and fibroblasts. The major host cell is the macrophage, where parasites multiply, eventually rupturing the cell and spread to the uninfected cells (reviewed in [5]). Infected monocytes and macrophages circulating in the peripheral blood are believed to be carriers of the parasite to distal sites [6]. In the dermis, parasites cause the cutaneous form of the disease (which can be localized or diffuse), whereas infection of the mucosa gives rise to mucocutaneous leishmaniasis. The metastatic spread of the infection to the spleen and liver results in visceral leishmaniasis. Although these are the major sites of visceral disease, parasites can also enter other organs, such as the bone marrow, lymph nodes, and lungs (reviewed in [5]). Presence of parasites in organs usually induces inflammation through cascade of signals that leads to recruitment of inflammatory cells, such as neutrophils, macrophages, eosinophils, and dendritic cells. These innate immune cells might phagocytose parasites and/or produce cytokines and chemokines that activate both innate and adaptive immune responses. Resulting responses can be protective and eliminate parasites, or ineffective and lead to chronic inflammation [7].

The sex of the host influences the incidence of disease, parasite burden, pathology, mortality, and immunological response against various parasites, including *Leishmania* both in humans and in rodents (reviewed in [7–12]).

In general, sex bias is observed after infection with *Leishmania* parasites, and men are more frequently infected than women ([13–15]; reviewed in [11, 12]), although in certain areas no sex bias in prevalence of disease was observed [16]. The higher susceptibility of males also applies to hamster [17] and mouse [18, 19]; reviewed in [12] models of leishmaniasis. The effect of male orchidectomy and female testosterone replacement studies suggests that the hormone testosterone can modulate systemic *L. major* infection in BALB/cAnPt, DBA/2N, DBA/2J, and F_1 hybrids (BALB/cAnPt x DBA/2N) mouse strains [18].

Importantly, the host genes, including those regulated differently in males and females, play a significant role in determining susceptibility and organ tropism for infectious diseases. Experimental data have shown different sex influence on susceptibility to relatively closely related pathogen species [20, 21], different sex biases in susceptibility to the same *Leishmania* species in different host genotypes [21, 22], and different sex and genetic influence on organ-specific pathology [21, 23, 24]. For example, high resistance to skin lesions induced by *L. mexicana* was observed in females but not in males of DBA/2 mice, but the sex effect was opposite in *L. major* infection [20].

Genotype influence on sex differences was defined in the studies of *L. major* infection [22, 24]. Giannini [22] found no sex effect on *L. major*-induced skin pathology and mortality in BALB/cJ mice, but a higher susceptibility of B10.129(10M)ScSn females than males. The comparison of *L. major* susceptibility in two strains, BALB/cHeA and CcS-11 [24], has shown that there is no significant sex influence on skin lesion development, splenomegaly, and hepatomegaly in these strains. However, parasite numbers in lymph nodes are higher in both BALB/c and CcS-11 males; moreover, CcS-11 males have higher parasite load in spleens, showing an organ-specific, sex-, and genotype-dependent pathology [24].

In the present study, we address influence of genotype and sex on infiltration of eosinophil leukocytes into the inguinal lymph nodes of *L. major*-infected mice. Eosinophils are granulocytes that develop in the bone marrow from pluripotent progenitors. They are released into the peripheral blood in phenotypically mature state and can be activated and recruited into tissues in response to appropriate stimuli, most notable IL-5, and the eotaxin chemokines [25].

Eosinophils contribute to the initiation of inflammatory and adaptive responses due to their bidirectional interactions with dendritic cells and T cells, as well as their large spectrum of secreted cytokines and soluble mediators. They have key immunoregulatory roles as professional antigen-presenting cells and modulators of functions of $CD4^+$ T cells, dendritic cells, B cells, mast cells, neutrophils, and basophils [26].

Eosinophil-associated disorders can affect practically all tissues and organs in the body, either individually or in combination. They are involved in inflammatory conditions affecting the skin, cardiovascular, nervous and renal system, gastrointestinal tract, and upper and lower airways [27, 28], are key effector cells in eosinophilic asthma [29], and their interaction with peripheral nerves has impact on pathology of many diseases. In addition, they are also involved in regulatory mechanisms modulating local and systemic immune responses and remodeling and repair mechanisms [30].

Eosinophils may have an important role in maintaining host survival in life-threatening viral infections [31]. They combat worms such as *Angiostrongylus cantonensis* [32], *Nippostrongylus brasiliensis* [33], *Litomosoides sigmodontis* [34], and *Brugia pahangi* [35]; but their role

in response to other nematoda is more complex. Eosinophils have no role in protection against *Schistosoma mansoni* [36]. They even promote larval growth in primary infection with *Trichinella spiralis* [37], but they mediate protective immunity against secondary infection with this nematode [38].

Activated eosinophils can kill [39] or support killing of *L. major* parasites [40]; however, in chronic disease, eosinophil infiltration might be a consequence of an ineffective elimination of these parasites and/or an excessive inflammatory response to the present pathogens [41].

Here, we analyzed genetic influence on eosinophil infiltration after *L. major* infection into the lymph nodes of strains BALB/cHeA (BALB/c), STS/A (STS), and selected 12 (out of 20) RC strains of CcS/Dem series [42]. Each of the 20 RC CcS/Dem strains contains a different unique set of approximately 12.5% genes of the donor strain STS on the genetic background of BALB/c. We found surprisingly high numbers of eosinophils in the inguinal lymph nodes of the strain CcS-9, males containing higher numbers of eosinophils than females. We analyzed genetics of this infiltration using microsatellite DNA markers and mapped four loci that control eosinophil numbers after *L. major* infection, one of them being strongly influenced by sex. We also found that the numbers of eosinophils in the lymph nodes correlate positively with the parasite load and that this correlation is partly genetically controlled and is higher in males than in females.

Methods

Mice

Tests of strain differences in eosinophil infiltration: Mice of the strains BALB/c (27 females, 27 males), STS (8 females, 9 males), CcS-1 (10 females, 13 males), CcS-3 (10 females, 10 males), CcS-4 (13 females, 12 males), CcS-5 (19 females, 27 males), CcS-7 (8 females, 12 males), CcS-9 (15 females, 10 males), CcS-11 (13 females, 13 males), CcS-12 (16 females, 12 males), CcS-15 (7 females, 12 males), CcS-16 (10 females, 13 males), CcS-18 (5 females, 3 males), and CcS-20 (13 females, 18 males) were infected with *L. major* as described previously [43, 44]. Mice were tested in eight successive experimental groups and were euthanized 8 weeks after infection. The age of mice at the time of infection was 7 to 47 weeks (mean 15 weeks, median 14 weeks).

A linkage study of eosinophil infiltration: F_2 hybrids between CcS-9 and BALB/c (age 11 to 21 weeks at the time of infection, mean and median age 14.8 and 15 weeks, respectively) were produced at the Institute of Molecular Genetics. When used for these experiments, the CcS-9 was in the 40th generation of inbreeding and therefore highly homozygous. Two hundred fifty-four F_2 hybrids between BALB/c and CcS-9 comprised 139 females and 115 males. Mice of the background parental strains BALB/c (18 females, 17 males) and STS (8 females, 6 males) and the RC strain CcS-9 (16 females, 14 males), 7 to 20 weeks old at the time of infection (mean 13 weeks, median 13 weeks), were used as controls. During the experiment, male and female mice were placed into separate rooms and males were caged individually. F_2 mice were tested in three independent experimental groups.

Ethical statement

All experimental procedures in this study comply with the Czech Government Requirements under the Policy of Animal Protection Law (No.246/1992) and with the regulations of the Ministry of Agriculture of the Czech Republic (No.207/2004), which are in agreement with all relevant European Union guidelines for work with animals and were approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics AS CR and by Departmental Expert Committee for the Approval of Projects of Experiments on Animals of the Academy of Sciences of the Czech Republic (permissions Nr. 274/2011; 89/2013).

Parasites

L. 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 [43]. 10^7 promastigotes from the passage, two cultivated for 6 days were inoculated in 50 μl sterile saline s.c. into mouse rump [44].

Disease phenotype

The size of the primary skin lesion was measured weekly using a Vernier caliper gauge. The mice were killed 8 weeks after infection and inguinal lymph nodes draining the site of infection were collected for further analysis.

Histological analysis

Inguinal lymph nodes of female and male mice were fixed in 10% neutral buffered formalin (NBF; approximately 4% formaldehyde) and embedded in paraffin using automatic tissue processor. Tissue sections (5–7 μm) were stained with hematoxylin, differentiated into 1% acid alcohol, stained with 1% alcoholic eosin, dehydrated, assembled with permanent mounting medium, and analyzed under a light microscope (Olympus BX51; Olympus Optical Co. (EUROPA) GMBH., Hamburg, Germany).

Eosinophil infiltration in the experiment with parental strains BALB/c, STS, and 12 RC strains was assessed using a semi-quantitative scoring system: 0, no eosinophil; 0.25, 1 eosinophil; 0.5, 2 eosinophils; 0.75, 3–4 eosinophils; 1, 5 eosinophils; 1.5, 6 eosinophils; 2, 7 eosinophils; 2.5, 8–9 eosinophils; 3, 10–15 eosinophils; and 4, more than 15 eosinophils per lymph node section (one section was used in experiment with parental strains BALB/c, STS and 12 RC strains).

In F_2 mice, as well as the parental strains BALB, STS, and CcS-9, eosinophil numbers were determined quantitatively. The total number of eosinophils was counted in the node section and each lymph node was assessed in four independent sections. The mean value of these four counts was used to calculate the role of genetic factors in control of eosinophil infiltration. Sixty slides from 15 mice were blindly recounted by an independent investigator with concordant results ($R = 0.913$, P value = 5.66×10^{-29}).

Genotyping of F_2 mice by PCR

DNA was isolated from tails using a standard proteinase procedure. The strain CcS-9 differs from BALB/c at STS-derived segments on eight chromosomes ([45] and unpublished results). These differential segments were typed in the F_2 hybrid mice between CcS-9 and BALB/c using 18 microsatellite markers (Research Genetics, Huntsville, FL, USA): D2Mit283, D2Mit148, D4Mit172, D4Mit23, D4Mit53, D4Mit17, D5Mit24, D5Mit143, D6Mit122, D6Mit274, D9Mit15, D11Mit141, D11Mit242, D11Nds18, D11Nds10, D16Mit19, D17Mit120, and D17Mit122. The markers were selected because their genomic location makes them suitable to detect linkage. The maximum distance between any two markers in the chromosomal segments derived from the strain STS or from the nearest BALB/c derived markers was 12.46 cM, and mean distance was 4.67 cM. The PCR genotyping for markers with fragment length difference more than 8 bp was performed using unlabeled primers as in [46, 47]. The PCR genotyping for markers with fragment length difference less than 8 bp was performed using [γ-^{32}P]ATP end-labeled primers as described elsewhere [48].

Measurement of parasite load in lymph nodes

Total DNA was isolated from the frozen lymph nodes, and parasite load was measured using PCR-ELISA according to the previously published protocol [49]. Briefly, for detection of *Leishmania* parasite DNA, in total DNA, PCR was performed using two primers (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 120-bp fragment within the conserved region of the kinetoplast minicircle of *Leishmania* parasite was amplified. In each PCR reaction, 50 ng of extracted total DNA was used. As a positive control, 20 ng of *L. major* DNA per reaction was amplified as a highest concentration of the standard. A 26-cycle PCR reaction was used for quantification of parasites. Parasite load was determined by measurement of the PCR product with the modified ELISA protocol (Pharmingen, San Diego, USA). The concentration of *Leishmania* DNA was measured at the ELISA Reader Tecan with the curve fitter program KIM-E (Schoeller Pharma, Prague, Czech Republic) using least squares-based linear regression analysis [24, 49].

Statistical analysis

The differences among BALB/c, STS, and CcS/Dem strains in eosinophil numbers in lymph nodes were evaluated by the analysis of variance (ANOVA) and Newman-Keuls multiple comparison test at 95% significance using the program Statistica for Windows 12.0 (StatSoft, Inc., Tulsa, OK, USA).

Differences between sexes in BALB/c, STS, and CcS/Dem strains were calculated by ANOVA (Statistica for Windows 12.0; StatSoft, Inc., Tulsa, OK, USA).

The role of genetic factors in control of eosinophil infiltration in F_2 hybrids was examined by ANOVA (Statistica for Windows 12.0; StatSoft, Inc., Tulsa, OK, USA). In order to obtain normal distribution of the analyzed parameter required for ANOVA, the obtained values were transformed as shown in the legends of tables. Markers and interactions with $P < 0.05$ were combined in a single comparison. In all ANOVA analyses strain or genotype, sex, and age were fixed factors, and the experiment was considered a random parameter.

For each independent variable, the partial R^2 was computed in the usual way by subtracting the regression sums of squares of the model without the variable (SS(b1,b2,b3,b4|b0)) of interest from the regression sums of squares of the full model (SS(b1,b2,b3,b4,b5|b0)); this difference divided by total regression sums of squares ((SS(b1,b2,b3,b4,b5|b0)):

$$\frac{(\mathrm{SS}(\mathrm{b1},\mathrm{b2},\mathrm{b3},\mathrm{b4}|\mathrm{b0})) - (\mathrm{SS}(\mathrm{b1},\mathrm{b2},\mathrm{b3},\mathrm{b4},\mathrm{b5}|\mathrm{b0}))}{(\mathrm{SS}(\mathrm{b1},\mathrm{b2},\mathrm{b3},\mathrm{b4},\mathrm{b5}|\mathrm{b0})}$$

indicated the contribution of the independent variable.

To obtain whole-genome significance values (corrected P values) the observed P values (aT) were adjusted according to Lander and Schork [50] using the formula:

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

where $G = 1.75$ Morgan (the length of the segregating part of the genome: 12.5% of 14 M); $C = 8$ (number of chromosomes segregating in cross between CcS-9 and BALB/c); $\rho = 1.5$ for F_2 hybrids; $h(T)$ = the observed statistics (F ratio).

The Spearman correlation coefficients between parasite numbers and eosinophil infiltration in the lymph nodes of F_2 hybrid mice were computed using the program Statistica for Windows 12.0 (StatSoft, Inc., Tulsa, OK, USA).

Results

Infiltration of eosinophils into the inguinal lymph nodes in parental strains BALB/c and STS and selected RC strains

We infected with *L. major* both females and males of the strains BALB/c, STS, and RC strains CcS-1, CcS-3, CcS-4, CcS-5, CcS-7, CcS-9, CcS-11, CcS-12, CcS-15,

CcS-16, CcS-18, and CcS-20 and used semi-quantitative scoring system to assess eosinophil infiltration (Table 1).

These studies showed mild and no infiltration into the lymph nodes of parental strains BALB/c (Fig. 1a, b) and STS (Fig. 1c, d), respectively. Strains CcS-9 ($P = 0.00020$) (Fig. 1e, f) and CcS-12 ($P = 0.0024$) exhibit significantly higher eosinophil infiltration in their lymph nodes than the background parental strain BALB/c. BALB/c and CcS-9 males presented higher eosinophil infiltration than females of these strains $P = 0.0089$ and $P = 0.016$, respectively. 80% of examined CcS-9 males in comparison with 26.67% of CcS-9 females contained infiltrating eosinophils, 50% of males having 7 and more eosinophils in their lymph nodes (Table 1). Sex difference in strains CcS-7,-11, and -18 was not significant. Strain CcS-9 with the highest eosinophil infiltration (Table 1) was selected for further genetic studies.

Four novel loci control eosinophil infiltration in leishmaniasis

We examined eosinophil numbers in lymph nodes in 254 F_2 hybrids between the strains BALB/c and CcS-9. The strain CcS-9 differs from BALB/c at STS-derived genetic regions located at eight chromosomes ([45], Šíma unpublished data). These differential STS-derived segments were genotyped in the F_2 hybrid mice using 18 microsatellite markers. A statistical analysis of linkage revealed four genetic loci that influence eosinophil infiltration into the inguinal lymph nodes after *L. major* infection.

Table 1 Eosinophil numbers in inguinal lymph nodes of *L. major*-infected mice

		% of mice with number of eosinophils (graded as 0–4) in section of inguinal lymph node									
		0	0.25	0.5	0.75	1	1.5	2	2.5	3	4
Strain	Sex	0	1	2	3–4	5	6	7	8–9	10–15	>15
BALB/c	Females	*100.00*	0	0	0	0	0	0	0	0	0
	Males	*81.48*	0	3.70	0	3.70	3.70	7.41	0	0	0
STS	Females	*100.00*	0	0	0	0	0	0	0	0	0
	Males	*100.00*	0	0	0	0	0	0	0	0	0
CcS-1	Females	*80.00*	0	0	0	10.00	0	10.00	0	0	0
	Males	*76.92*	7.69	0	0	15.38	0	0	0	0	0
CcS-3	Females	*100.00*	0	0	0	0	0	0	0	0	0
	Males	*90.00*	0	0	0	10.00	0	0	0	0	0
CcS-4	Females	*92.31*	0	7.69	0	0	0	0	0	0	0
	Males	*91.67*	0	0	0	0	0	0	0	8.33	0
CcS-5	Females	*100.00*	0	0	0	0	0	0	0	0	0
	Males	*96.30*	0	0	0	3.70	0	0	0	0	0
CcS-7	Females	*100.00*	0	0	0	0	0	0	0	0	0
	Males	*75.00*	0	0	0	25.00	0	0	0	0	0
CcS-9	Females	73.33	0	0	0	20.00	0	6.67	0	0	0
	Males	20.00	0	10.00	0	20.00	0	10.00	20.00	20.00	0
CcS-11	Females	*84.62*	0	7.69	7.69	0	0	0	0	0	0
	Males	61.54	0	23.08	0	7.69	0	7.69	0	0	0
CcS-12	Females	56.25	0	0	0	31.25	6.25	0	0	6.25	0
	Males	50.00	0	8.33	0	16.67	0	16.67	0	0	8.33
CcS-15	Females	*100.00*	0	0	0	0	0	0	0	0	0
	Males	*91.67*	0	8.33	0	0	0	0	0	0	0
CcS-16	Females	*90.00*	0	10.00	0	0	0	0	0	0	0
	Males	*100.00*	0	0	0	0	0	0	0	0	0
CcS-18	Females	*100.00*	0	0	0	0	0	0	0	0	0
	Males	33.33	0	66.67	0	0	0	0	0	0	0
CcS-20	Females	*92.31*	0	0	0	7.69	0	0	0	0	0
	Males	*95.00*	0	0	0	5.00	0	0	0	0	0

Eosinophil numbers in lymph nodes depending on genotype and sex. Eosinophil infiltration was evaluated as described in the "Methods" section.
Numbers higher than 75% are shown in italics

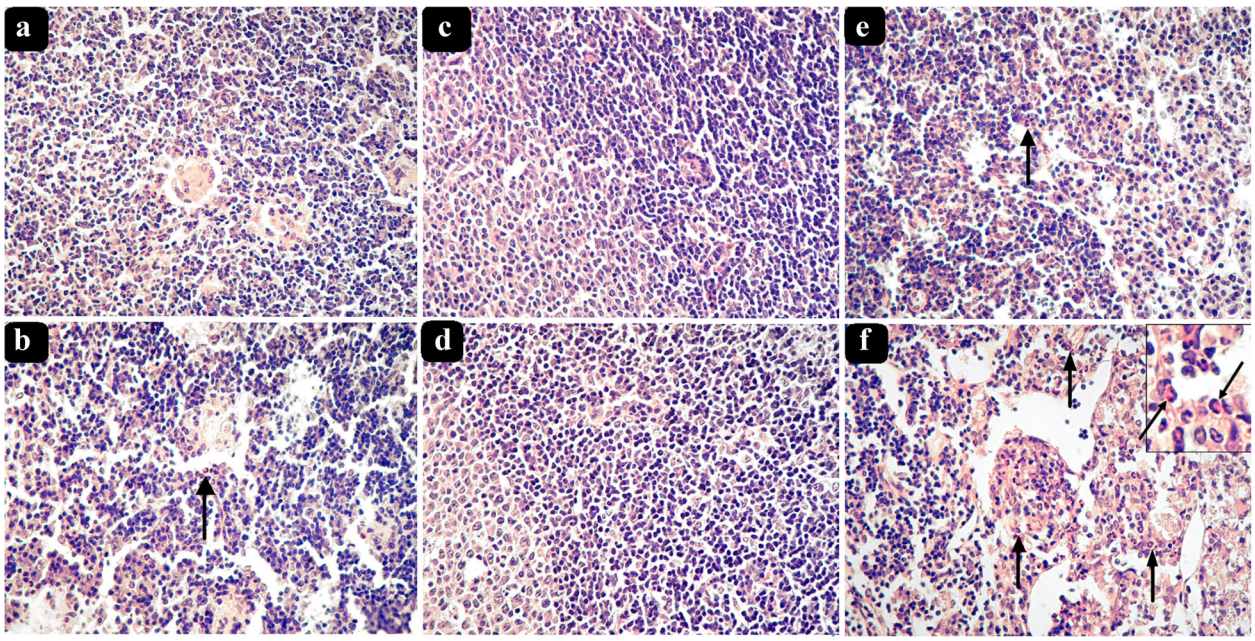

Fig. 1 Eosinophils in hematoxylin-eosin-stained inguinal lymph node sections of *L. major*-infected female and male mice. **a** BALB/c female, **b** BALB/c male, **c** STS female, **d** STS male, **e** CcS-9 female, and **f** CcS-9 male with detail of eosinophils. *Arrows* show positions of eosinophils

The effects of *Lmr14* (*L. major* response 14) linked to D2Mit283 (corrected *P* value = 0.0081) and *Lmr25* linked to D5Mit143 (corrected *P* value = 0.044) were detectable and significantly independent of each other or other genes (the main effects) (Table 2). *Lmr14* operated only in males (corr. *P* value of marker and sex interaction =0.0085)(Table 2, Fig. 2), higher numbers of eosinophils were associated with presence of BALB/c (C) allele (Fig. 2c). The *P* value for *Lmr14* was significant only in cross (CcS-9 × BALB/c)F_2 (where the mother of the F_1 hybrids was CcS-9 and the father was BALB/c) (Fig. 2c), but not in cross (BALB/c × CcS-9)F_2 (where the mother was BALB/c and the father was CcS-9) (Fig. 2d). However, interaction between the cross and marker D2Mit283 was not significant (corr. $P = 0.6$). The effect of *Lmr25* was not influenced by sex, and higher numbers of eosinophils were observed in heterozygotes (Table 2).

In contrast to the main effects of *Lmr14* and *Lmr25*, *Lmr15* (linked to D11Nds10) and *Lmr26* (linked to D9Mit15) operated in cooperation with each other (non-additive, epistatic, interaction) (corrected $P = 0.010$). F_2 male mice of the cross (BALB/c × CcS-9)F_2 with homozygous BALB/c (CC) alleles at both *Lmr26* and *Lmr15* had nearly nine times higher numbers of eosinophils in the lymph nodes than mice with homozygous STS (SS) alleles at both these loci, and nearly 90 times higher than mice with homozygous CC alleles at *Lmr26* and CS alleles at *Lmr15* (Table 3). The linkage was detected only in males, but the interaction between sex and marker was not significant (corr. $P = 0.19$).

Positive correlation between parasite numbers and eosinophils in the inguinal lymph nodes

We have determined parasite load in the lymph nodes of the F_2 hybrids between BALB/c and CcS-9 and analyzed the relationship between parasite numbers in lymph nodes and eosinophil infiltration to this organ. In both sexes pooled, there was a positive correlation between parasite numbers and eosinophil infiltration $R = 0.39$, $P = 1.3 \times 10^{-10}$, and the correlation was significant in males $R = 0.29$, $P = 0.0017$, but not in females $R = 0.14$, $P = 0.10$. This correlation is at least partly controlled by *Lmr* loci, because in F_2 hybrid mice, this correlation was positive in male homozygous for the *Lmr14* (D2Mit283) BALB/c allele (CC) ($R = 0.51$, $P = 0.016$) and STS allele (SS) ($R = 0.50$, $P = 0.00088$), but no correlation was observed in heterozygotes ($R = -0.013$, $P = 0.92$).

Discussion

Eosinophil infiltration in strain CcS-9 exceeds that of both parents

Strain CcS-9 that contains a set of approximately 12.5% genes of the donor strain STS and 87.5% genes of the background strain BALB/c exhibited numbers of infiltrating eosinophils (Fig. 1, Table 1) exceeding those in both parental strains BALB/c and STS. The observations of progeny having a phenotype, which is beyond the range of the phenotype of its parents, are not rare in traits controlled by multiple genes. It was detected in different tests of immune responses of RC strains in vitro [51–56] and in vivo [21, 57–60], and in analysis of

Table 2 Main effect of loci that control eosinophil numbers in the inguinal lymph nodes of *L. major*-infected F_2 hybrids between CcS-9 and BALB/c

Locus	Group	Marker	Genotype									*P* value	Corr. *P* value	% of expl. var.
			CC			CS			SS					
Lmr14	Both sexes	D2Mit283	**2.62**	1.229	±0.003 (*n* = 57)	**3.62**	1.234	±0.002 (*n* = 111)	**2.67**	1.229	±0.002 (*n* = 74)	NS	NS	NA
	Females		**1.24**	1.217	±0.003 (*n* = 35)	**1.45**	1.220	±0.002 (*n* = 61)	**1.62**	1.221	±0.003 (*n* = 32)	NS	NS	NA
	Males both crosses		**5.15**	1.239	±0.005 (*n* = 22)	**8.94**	1.247	±0.003 (*n* = 50)	**4.02**	1.236	±0.004 (*n* = 41)	5.5×10^{-2}	NS	NA
	Males (BALB/c × CcS-9)F_2		**3.37**	1.233	±0.006 (*n* = 15)	**9.09**	1.242	±0.004 (*n* = 32)	**3.78**	1.235	±0.005 (*n* = 27)	NS	NS	NA
	Males (CcS-9 × BALB/c)F_2		**13.63**	1.253	±0.005 (*n* = 7)	**18.34**	1.257	±0.004 (*n* = 18)	**4.51**	1.237	±0.005 (*n* = 14)	1.08×10^{-4}	$\mathbf{8.11 \times 10^{-3}}$	**36.22**
Lmr25	Both sexes	D5Mit143	**2.07**	1.225	±0.003 (*n* = 66)	**4.33**	1.237	±0.002 (*n* = 107)	**1.96**	1.225	±0.003 (*n* = 67)	9.53×10^{-4}	$\mathbf{4.36 \times 10^{-2}}$	**5.02**
	Females		**1.09**	1.215	±0.003 (*n* = 37)	**2.04**	1.225	±0.002 (*n* = 56)	**1.20**	1.216	±0.003 (*n* = 36)	6.3×10^{-3}	NS	NA
	Males		**5.71**	1.241	±0.004 (*n* = 31)	**7.40**	1.244	±0.003 (*n* = 51)	**4.41**	1.237	±0.004 (*n* = 31)	NS	NS	NA

Mean and SE values were obtained by analysis of variance. In order to obtain normal distribution required for analysis of variance, the value of eosinophil numbers in the inguinal lymph nodes was transformed by using the 0.1th power of natural logarithm of the (observed value ×1000). The numbers in bold give the average non-transformed values. C and S indicate the presence of BALB/c and STS allele, respectively
n number of mice

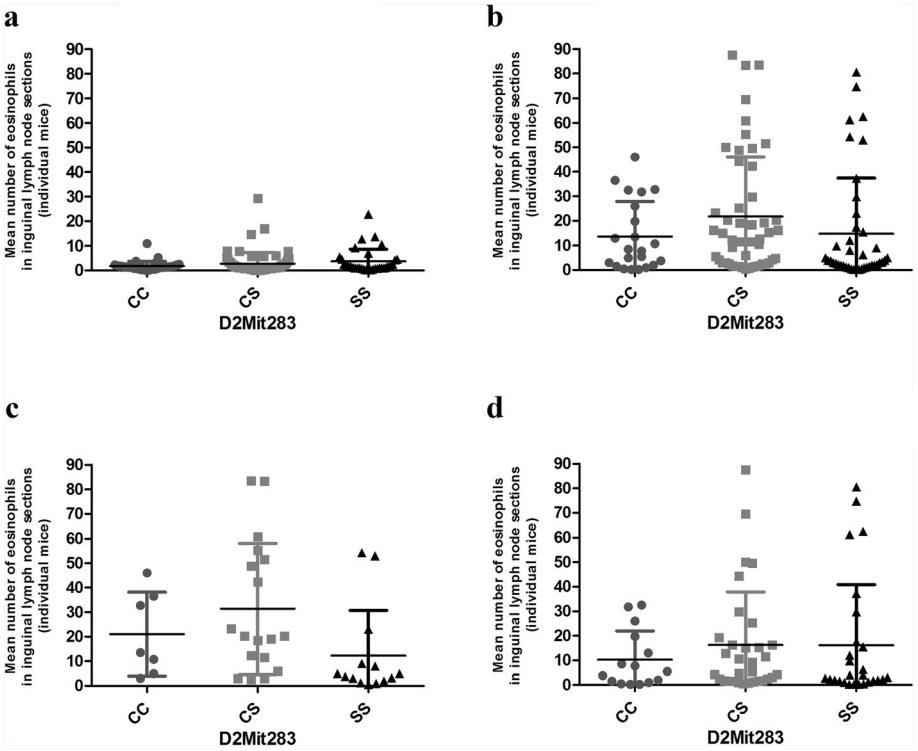

Fig. 2 Effects of genotype and sex on eosinophil infiltration at *Lmr14* (D2Mit283) **a** females (corr. *P* = NS), **b** males (corr. *P* = NS), **c** males (CcS-9 × BALB/c)F_2 cross (corr. $P = 8.11 \times 10^{-3}$), and **d** males (BALB/c × CcS-9)F_2 cross (corr. *P* = NS).These data are shown for sex and genotype CC—BALB/c homozygotes, CS—heterozygotes, SS—STS homozygotes as mean ± SD. *NS* not significant

Table 3 Interaction between loci controlling eosinophil numbers in the inguinal lymph nodes in *L. major*-infected F_2 hybrids between CcS-9 and BALB/c

		D9Mit15 (**Lmr26**)								
		CC			CS			SS		
		$P = 3.7 \times 10^{-2}$			Corr. P = NS			% of expl. var. = NA		
D11Nds10 (**Lmr15**)	CC	**4.24**	1.236	±0.006	**3.03**	1.231	±0.004	**2.19**	1.226	±0.005
Both sexes				($n = 13$)			($n = 26$)			($n = 17$)
	CS	**1.45**	1.220	±0.004	**3.76**	1.235	±0.003	**3.88**	1.235	±0.003
				($n = 21$)			($n = 63$)			($n = 39$)
	SS	**2.56**	1.229	±0.005	**2.37**	1.228	±0.004	**3.36**	1.233	±0.005
				($n = 17$)			($n = 30$)			($n = 16$)
		$P = 0.67$			Corr. P = NS			% of expl. var. = NA		
D11Nds10 (**Lmr15**)	CC	**1.89**	1.224	±0.007	**1.60**	1.221	±0.004	**1.42**	1.219	±0.005
Females				($n = 6$)			($n = 18$)			($n = 10$)
Both crosses	CS	**1.37**	1.219	±0.005	**1.53**	1.220	±0.003	**2.28**	1.227	±0.005
				($n = 11$)			($n = 38$)			($n = 17$)
	SS	**1.67**	1.222	±0.007	**1.46**	1.220	±0.004	**1.06**	1.214	±0.007
				($n = 7$)			($n = 16$)			($n = 6$)
		$P = 2.63 \times 10^{-4}$			Corr. $P = \mathbf{1.037 \times 10^{-2}}$			% of expl. var. = **15.35**		
D11Nds10 (**Lmr15**)	CC	**11.71**	1.251	±0.010	**12.05**	1.251	±0.008	**3.49**	1.234	±0.008
Males				($n = 7$)			($n = 8$)			($n = 7$)
Both crosses	CS	**1.06**	1.214	±0.007	**23.15**	1.260	±0.005	**12.22**	1.251	±0.004
				($n = 10$)			($n = 25$)			($n = 22$)
	SS	**6.55**	1.243	±0.008	**5.18**	1.239	±0.005	**7.27**	1.244	±0.007
				($n = 10$)			($n = 14$)			($n = 10$)
		$P = 0.41$			Corr. P = NS			% of expl. var. = NA		
D11Nds10 **(Lmr15)**	CC	**10.37**	1.249	±0.008	**12.60**	1.252	±0.006	**6.78**	1.243	±0.008
Males				($n = 2$)			($n = 2$)			($n = 3$)
Cross CcS-9 × BALB	CS	**8.49**	1.246	±0.022	**21.20**	1.258	±0.004	**9.85**	1.248	±0.007
				($n = 2$)			($n = 10$)			($n = 10$)
	SS	**6.92**	1.244	±0.012	**3.75**	1.235	±0.007	**13.88**	1.253	±0.022
				($n = 4$)			($n = 4$)			($n = 2$)
		$P = 3.97 \times 10^{-4}$			Corr. $P = 1.629 \times \mathbf{10^{-2}}$			% of expl. var. = **21.19**		
D11Nds10 (**Lmr15**)	CC	**44.01**	1.267	±0.012	**32.78**	1.264	±0.010	**8.88**	1.247	±0.016
Males				($n = 5$)			($n = 6$)			($n = 4$)
Cross BALB × CcS-9	CS	**0.47**	1.199	±0.010	**13.04**	1.252	±0.006	**12.40**	1.252	±0.006
				($n = 8$)			($n = 15$)			($n = 12$)
	SS	**9.73**	1.248	±0.011	**6.78**	1.243	±0.007	**4.84**	1.238	±0.008
				($n = 6$)			($n = 10$)			($n = 8$)

Mean and SE values were obtained by analysis of variance. In order to obtain normal distribution required for analysis of variance value of eosinophil numbers in serum inguinal lymph nodes was transformed by using the 0.1th power of natural logarithm of the (observed value ×1000). The numbers in bold give the average non-transformed values. C and S indicate the presence of BALB/c and STS allele, respectively
n number of mice

expression quantitative trait loci (QTLs) from the livers of chromosome substitution strains [61]. These observations are due to multiple gene-gene interactions of QTLs, which in new combinations of these genes in RC or chromosomal substitution strains can lead to the appearance of new phenotypes that exceed their range in parental strains. In addition, with traits controlled by multiple loci, parental strains often contain eosinophil high infiltration alleles at

some of them and eosinophil low infiltration alleles at others, and some progeny may receive predominantly eosinophil high infiltration alleles from both parents.

Sex influence on eosinophil infiltration

Our data show a sex influence on eosinophil numbers in the inguinal lymph nodes. Differences between the immune system of females and males have been well documented [62–64] and could result in differences in susceptibility to diseases with immune component. Immune responses including those involving eosinophils might be modulated by steroid hormones [65, 66]. Moreover, some of the differences between females and males might be due to sex-specific genetic architecture, characterized by profound gene-sex interactions [67, 68]. This would mean that some genes controlling response to *L. major* might operate differently in the two sexes. Indeed, locus *Lmr14* controls eosinophil infiltration only in males. Genes controlling infections that appear to be sex dependent have been observed also with other infectious agents such as viruses [69–71], bacteria [72], parasites [58], and fungi [73] and helminths [74]. Some of sex-dependent QTLs exhibit a higher or exclusive influence on susceptibility in females [58, 69, 71–73] or males [69, 71–74], phenotypic effect of other genes is present in both sexes, but with opposite direction of effect [69, 70]. All these reported loci are situated on autosomal chromosomes. In contrast to the sex chromosomes, the autosomal genome is shared by both sexes. However, although the DNA sequence, gene structure, and frequency of polymorphism on the autosomes do not differ between males and females, the regulatory genome is sexually dimorphic [68].

Future genetic and functional studies will help to establish the mechanistic basis of the observed gene-sex interactions.

Loci controlling eosinophil infiltration and other immune traits

The *Lmr* loci influencing eosinophil infiltration may be related to QTLs that determine certain immunologically relevant traits, because they co-map with other immunological functional polymorphisms.

Interestingly, two of the eosinophil controlling loci, *Lmr15* and *Lmr26* co-localize with loci that determine hemopoietic cell cycling measured by cobblestone area-forming cell (CAFC) assay using cells from the bone marrow [75]. *Lmr15* encompasses the mouse ortholog of human gene *IL5*, whose polymorphism was found to be associated with eosinophil counts in the blood [76], and *Lmr26* co-localizes also with locus *Tria5* that modifies in vitro proliferation of mouse splenocytes stimulated by soluble anti-CD3 [77].

The four described loci comprise several genes (Fig. 3), whose biological function is compatible with the effects on eosinophil infiltration [78–90] and their potential role can now be investigated. However, the effects of these *Lmr* loci might be also caused by genes that are at the present not considered as candidates. The issue of identity of eosinophil controlling genes and their possible relationship to other immune traits will be resolved by a recombinational analysis.

The positive correlation between eosinophil infiltration and parasite load suggests that the observed eosinophilic infiltration reflects ineffective inflammation. This is in

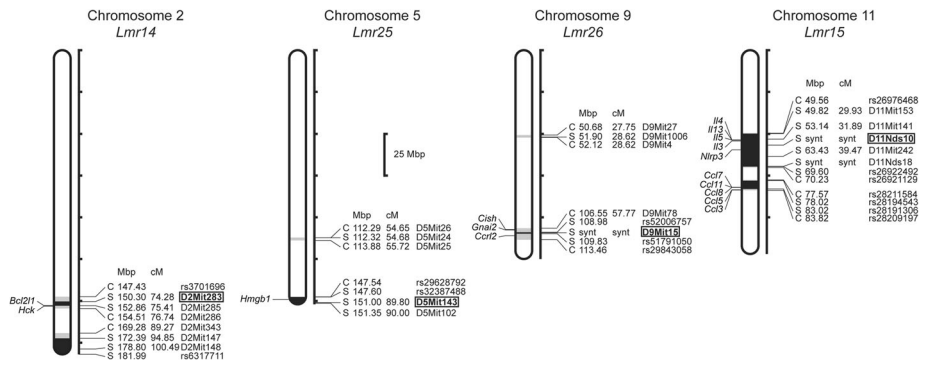

Fig. 3 Loci controlling eosinophil numbers in CcS-9. The regions of STS and BALB/c origins are represented as *dark* and *white*, respectively; the boundary regions of undetermined origin are *shaded*. *C* and *S* indicate the presence of BALB/c and STS allele, respectively. Only the markers and SNPs defining the boundaries the STS-derived segment and the markers that were tested for linkage are shown. The markers that exhibit significant *P* values (genome-wide corrected) are shown in *bold*. *Lmr* loci on chromosomes 2 and 11 detected in a cross between CcS-9 and BALB/c overlap with loci *Lmr14* [91–93] and *Lmr15* [91, 93] detected in cross of CcS-16 with BALB/c, and were therefore given the same name. Abbreviations show genes that have been reported to be involved in eosinophil functions: *Bcl2l1* (BCL2-like 1) [78], *Ccl11* (chemokine (C-C motif) ligand 11) [79] *Ccl3* (chemokine (C-C motif) ligand 3) [79], *Ccl5* (chemokine (C-C motif) ligand 5) [79], *Ccl7* (chemokine (C-C motif) ligand 7) [79], *Ccl8* (chemokine (C-C motif) ligand 8) [80], *Ccrl2* (chemokine (C-C motif) receptor-like 2) [81], *Cish* (cytokine inducible SH2-containing protein) [82], *Gnai2* (guanine nucleotide binding protein (G protein), alpha inhibiting 2) [83], *Hck* (hemopoietic cell kinase) [84], *Hmgb1* (high mobility group box 1) [85], *Il13* (interleukin 13) [86], *Il3* (interleukin 3) [87], *Il4* (interleukin 4) [88], *Il5* (interleukin 5) [89], and *Nlrp3* (NLR family, pyrin domain containing 3) [90]. However, the effects of eosinophil controlling loci might be caused by genes that are at the present not considered as candidates

agreement with kinetic studies showing that parasite presence preceded presence of infiltrating cells including eosinophils. This infiltration was higher in mice that were unable to control infection [41].

We have found positive correlation between eosinophil infiltration and parasite numbers in *Lmr14* in homozygous (CC or SS), but not in heterozygous (CS) F_2 hybrid males. The lack of positive correlation between eosinophil infiltration and parasite load in *Lmr14* heterozygotes (CS) may reflect a more effective inflammation process, perhaps facilitated by other phenotypic effects of *Lmr14* that include circulating levels of IFNγ, TNF, IgE, and IL-12 [91] and possibly other as yet undetected regulatory effects. This possibility has to be tested in future experiments.

Conclusions

This is the first demonstration of genetic loci and sex influence controlling infiltration of eosinophils into the lymph nodes and its relationship with parasite load. Some of these loci comprise genes with broader biological and immunological effects, so they might be relevant also in control of other diseases and symptoms mediated by eosinophils.

Our data also suggest that ignoring sex in gene mapping might prevent detection of sex-dependent QTLs.

Abbreviations

CcS/Dem: Series of recombinant congenic strains derived from the mouse donor strain STS/A (STS) and the background strain BALB/cHeA (BALB/c); QTL: Quantitative trait locus; RCS: Recombinant congenic strains

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Availability of data and materials

The datasets collected and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MSl helped to conceive the study, performed animal experiments with RC strains, carried out all histological analyses, and contributed to the writing of the manuscript. VV performed statistical analyses and contributed to the writing of the manuscript. MC carried out animal experiments with F_2 hybrids, typed F_2 hybrids, and contributed to the writing of the manuscript. TK estimated parasite load in lymph nodes and re-counted eosinophils in number of mice. MŠí helped with typing of F_2 hybrids, defined precisely STS-derived segments of strain CcS-9, and contributed to the writing of the manuscript. MSv cultivated parasites and helped with parasitology experiments. PD helped to analyze the data and contributed to the writing of the manuscript. ML conceived the study, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable. This study did not include human subjects.

Ethics approval

Not applicable. This study did not include human subjects.

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4.4 Genotyping of short tandem repeats (STRs) markers with 6 bp or higher length difference using PCR and high resolution agarose electrophoresis

Many STR markers have length difference 6 bp or higher and can be typed using simple and quick method, which include DNA isolation either Proteinase K or NaOH protocol followed by PCR and high resolution agarose electrophoresis. Results can be read from agarose gel by comparing positions of PCR product bands of known size and samples, where size of the DNA fragment should be estimated. In this technical report, we described this method in details including troubleshooting advices. This method was used for genotyping of mice in studies presented in this thesis.

protocol exchange

Protocol Exchange (2015)
http://www.nature.com/protocolexchange/protocols/3973

Genotyping of short tandem repeats (STRs) markers with 6 bp or higher length difference using PCR and high resolution agarose electrophoresis

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This protocol describes DNA typing of short tandem repeats (STRs), which differ in at least 6 bp using PCR and optimized high resolution electrophoresis. DNA preparation by NaOH method or by standard TRI reagent procedure takes about 4 hours. When DNA is prepared in master plates, a single person can test 192 samples in maximally 5 hours. It is suitable for quick test of interval-specific congenic strains, marker-assisted breeding of congenic mouse strains, test of presence of transgenes, knock-out or knock-in alleles in gene targeting technologies in breeding crosses, and for genotyping of intraspecific crosses, especially those derived from parents, which differ in limited percentage of their genomes.

KEYWORDS Gene typing; STR (short tandem repeat); microsatellite; allele detection; PCR; electrophoresis; high resolution.

INTRODUCTION

Short tandem repeats (STRs) or microsatellites occur in the form of iteration of repeat units from a single base pair (bp) to thousands of bp. Mono-, di-, tri- and tetranucleotide repeats are the main types of microsatellites, but repeats of five (penta-) or six (hexa-) nucleotides are usually classified as microsattelites as well. Dinucleotide repeats dominate, $(CA)_n$ repeats are most frequent, followed by $(AT)_n$, $(GA)_n$, and $(GC)_n$, the last type or repeat being rare. Large majority of simple repeats are embedded in non-coding DNA, either in the intergenic sequences or in the introns[1], but approximately 17% of human genes contain STRs in their open reading frames[2] including promoters[3].

Eukaryotic genomes contain a large number of STRs[4,5]; repetitive sequences are present in low numbers also in prokaryotes[6]. Microsatellites are among the most variable types of DNA sequence in the genome. Their polymorphism is derived mainly from variability in length. Thus, abundance of microsatellites in combination with their polymorphism and hypervariability, and possibility to amplify them by polymerase chain reaction (PCR) allows to use them for the construction of high-density genetic maps and enables the molecular tagging of genes[7-12] and a wide use in genetics of susceptibility to diseases, breeding and population studies, gene expression analysis, forensics, diagnostics and pathogen detection and classification, and many other applications.

Microsatellites can be identified from sequence data with the use of computational tools such as SPUTNIK (Abajian 1994, http://abajian.net/sputnik/ - original webpage, no longer functional), TRF (Tandem Repeats Finder)[13], SSRIT (Simple Sequence

Repeat Identification Tool)[14], TROLL (Tandem Repeat Occurrence Locator)[15], MicroSAtellite (MISA, http://pgrc.ipk-gatersleben.de/misa/)[16], WebSat (http://wsmartins.net/websat/)[17], GMATo (Genome-wide Microsatellite Analyzing Tool)[18], and MsDetector[19]. Flanking DNA sequences may then be analysed for the presence of suitable forward and reverse PCR primers to assay the STR loci. Several computational tools are currently available for the identification of STRs within sequence data as well as for the design of PCR primers suitable for the amplification of specific loci[20-22] (http://www.ufpel.edu.br/[23]).

Information about STR sequences can be obtained also from public databases. Information about human sequences is available in STRBase (http://www.cstl.nist.gov/strbase/)[24], data about human, mouse, dog, rat and chicken STRs linked with SNP are in SNPSTR database (http://www.sbg.bio.ic.ac.uk/~ino/SNPSTRdatabase.html)[25]. Mouse microsatellites and primers flanking each repeat are listed in Mouse Microsatellite Database of Japan (http://www.shigen.nig.ac.jp/mouse/mmdbj/top.jsp), and in Mouse Genome Informatics (http://www.informatics.jax.org/marker/).

We have optimized analysis of PCR product in agarose electrophoresis using the mix of 4:1 of Methaphore (Cambrex) and UltraPure™ (Invitrogen) Agarose, respectively. This allowed us to separate PCR products with 6 and more bp length difference. Method was successfully used to map mouse genes controlling susceptibility to *Leishmania major*[26], *Trypanosoma brucei brucei*[27] and *Leishmania tropica*[28]. Quality of DNA obtained by NaOH extraction is suitable for typing of majority of markers, and then whole procedure can be

performed within one day (Figure 1). In case of need of higher DNA quality, extraction can be performed using TRI reagent (4 hours) or Proteinase K (three days).

The present procedure is cheap and quick and it is suitable for test of interval-specific congenic strains, marker-assisted breeding of congenic mouse strains, test of presence of transgenes, knock-out or knock-in alleles in segregating experimental and breeding crosses, and for typing of intraspecific crosses, especially those derived from parents, which differ in limited percentage of their genomes.

MATERIALS

REAGENTS

DNA preparation

Option (A) Isolation of DNA by NaOH

- **NaOH** (BDH, cat. no. 10438) **! 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.
- **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.
- **HCl** (Sigma-Aldrich, cat. no. H1758) **! 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.
- **Sterile distilled H_20**

Option (B) DNA isolation using TRI reagent

- **TRI reagent** (Sigma-Aldrich, cat. no. T9424) **! 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.
- **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.
- **Ethanol** (PENTA, cat. no. 32294) **! CAUTION** Highly flammable. Keep the container tightly closed. Keep away from sources of ignition—no smoking.
- **Sodium citrate tribasic hydrate** (Sigma-Aldrich, cat. no. 25114)
- **NaOH** (BDH, cat. no. 10438) **! 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.
- **Sterile distilled H_20**

Option (C) Isolation of DNA by proteinase K

- **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) **! CAUTION** irritant, cytotoxic and weakly genotoxic.
- **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.
- **NaCl** (Sigma-Aldrich, cat. no. S7653)
- **NaOH** (BDH, cat. no. 10438) **! 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.
- **HCl** (Sigma-Aldrich, cat. no. H1758) **! 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.

- **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.
- **Proteinase K** (Sigma-Aldrich, cat. no. P6556)
- **Sterile distilled H_20**

PCR reaction

- **$MgCl_2$** (Sigma-Aldrich, cat. no. 208337): **! CAUTION** Do not breathe dust. Avoid contact with skin and eyes.
- **REDTaq® DNA Polymerase** (Sigma-Aldrich, cat. no. D4309)
- **10x REDTaq® PCR Reaction Buffer** (Sigma, cat. no. B5926)
- **Forward and reverse primer** (Research Genetics, Generi Biotech)
- **dNTPs** (Sigma-Aldrich, cat. no. DNTP 100A-1KT) **! CAUTION** May cause irritation to skin, eyes, and respiratory tract, may affect kidneys.
- **Sterile distilled H_20**

Agarose electrophoresis

- **Agarose MetaPhor** (Cambrex, cat. no. Lonza 50184) **! CAUTION** Irritant, protect from eyes and skin contact.

- **Agarose UltraPure™** (Invitrogen, cat. no. 16500500) **! CAUTION** Irritant, protect from eyes and skin contact.
- **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.
- **Boric acid** (Serva, cat. no. 15165) **! CAUTION** Hazardous in case of skin contact (irritant), of eye contact (irritant), of ingestion, of inhalation. Slightly hazardous in case of skin contact (permeator). The substance may be toxic to kidneys, cardiovascular system, central nervous system (CNS). Repeated or prolonged exposure to the substance can produce target organs damage. It may affect fertility or cause damage to the unborn child.
- **EDTA-disodium** (SERVA Electrophoresis, cat. no. 11280; pH 8.0) **! CAUTION** irritant, cytotoxic and weakly genotoxic.
- **HCl** (Sigma-Aldrich, cat. no. H1758) **! 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.
- **NaOH** (BDH, cat. no. 10438) **! 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.

- **Ethidium bromide** (Calbiochem, cat. no. 331565) **! CAUTION** Material may be harmful by all routes of entry; inhalation, ingestion, or skin absorption. Material causes eye and skin irritation and is irritating to mucous membranes and upper respiratory tract. This agent intercalates DNA strands and was mutagenic in a number of test systems (yeast cells). The chemical, physical, and toxicological properties have not been thoroughly investigated in humans.
- **Orange G** (Lachema, cat. no. 30699)
- **Glycerol** (Lachema, cat. no. 30217)
- **50 bp DNA ladder** (Biolabs, cat. no N3236L)
- **Distilled H_20**

EQUIPMENT

DNA preparation

Option (A) Isolation of DNA by NaOH

- **Water bath** (Julabo SW 22)
- **Centrifuge** (Eppendorf - Model 5810 R)
- **NanoDrop Spectrophotometer** (ND-1000, Thermo Scientific)

Option (B) DNA isolation using TRI reagent

- **Polytron®** (Kinematica - PT 2100 Homogenizers)
- **Minicentrifuge** (Eppendorf® MiniSpin)
- **Centrifuge** (Eppendorf - Model 5810 R)
- **NanoDrop Spectrophotometer** (ND-1000, Thermo Scientific)

Option (C) Isolation of DNA by proteinase K

- **Water bath** (Julabo SW 22)
- **Centrifuge** (Eppendorf - Model 5810 R)
- **Disposable inoculation loops** (P-Lab, cat. no. K002650)
- **NanoDrop Spectrophotometer** (ND-1000, Thermo Scientific)

PCR reaction

- **96-well PCR plate** (AB-gene, cat. no. AB-0600)
- **Sealing Tape** (Nunc, cat. no. 236707) / **PCR 8 strip flat caps** (AB-gene, AB-0784)
- **DNA Engine Dyad® Peltier Thermal Cycler** (Bio-Rad)

Agarose electrophoresis

- **Electrophoresis equipment** (Shelton Scientific – Model JSB-96)
- **Bio imaging system** (Syngene)

REAGENT SETUP

DNA preparation

Option (A) Isolation of DNA by NaOH

50 mM NaOH

Add 1 g of NaOH to 500 ml of sterile distilled H_2O. Can be stored at room temperature for years.

1M Tris, pH 8.0

Add 12.11 g of Tris to approx. 60 ml of sterile distilled H_2O, adjust pH to 8.0 with HCl, adjust volume to 100 ml. Can be stored at room temperature for years.

Option (B) DNA isolation using TRI reagent

8 mM NaOH

Add 0.16 g of NaOH to 500 ml of sterile distilled H_2O. Can be stored at room temperature for years.

DNA washing solution

100 mM sodium citrate tribasic hydrate in 10% (vol/vol) ethanol. Usually mix 13 g of sodium citrate tribasic hydrate with 500 ml of 10% ethanol.

Option (C) Isolation of DNA by proteinase K

Lysis buffer

Comprises 100 mM Tris, 5 mM EDTA-disodium (pH 8.0), 0.2% SDS and 200 mM NaCl. For preparation of 1 liter of lysis buffer, mix 10 ml 20% (wt/vol) SDS, 12.11 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 186.15 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 with HCl; adjust volume to 1 liter with distilled water. Solution can be stored at room temperature for up to 1 year.

Proteinase K

Prepare 500 µl aliquots with Proteinase K concentration 20 mg/ml. Can be stored at -20°C for several years.

PCR reaction

5x PCR buffer total for REDTaq® DNA Polymerase

5x PCR buffer total for REDTaq® DNA Polymerase should contain 50 mM Tris-HCl (pH 8.3), 250 mM KCl, 7.5 mM $MgCl_2$ ▲ **CRITICAL STEP** The concentration of $MgCl_2$ must be exact, 1 mM dNTP, and 0,05% Gelatin.

For 50 ml of 5x PCR buffer total mix 25 ml of 10x REDTaq® PCR Reaction Buffer, 500 µl of each 100 mM dNTPs (dATP, dCTP, dGTP, dTTP), 2 ml of 50 mM $MgCl_2$ (▲ **CRITICAL STEP** Optimal concentration of $MgCl_2$ for certain primers might differ) and 21 ml sterile distilled H_2O. Make 750 µl aliquots. Can be stored at -20°C for up to 2 years. To prepare 50 mM $MgCl_2$ add 0.24 g to 50 ml of sterile distilled water.

Use 10x REDTaq® PCR Reaction Buffer supplied with polymerase or prepare yourself. 10x PCR buffer contains 100 mM Tris-HCl (pH 8.3, SERVA Electrophoresis, cat. no. 37190, Sigma-Aldrich, cat. no. H1758), 500 mM KCl (Sigma-Aldrich, cat. no. P9333), 11 mM $MgCl_2$ and 0.1% gelatin (Sigma-Aldrich, cat. no. G9391), can be stored at -20°C for years.

Primers

Research Genetics primers has suitable concentration 6.6 µM. Generi Biotech sells lyophilized primers, which are dissolved to concentration 0.1 mM and they have to be diluted to 6.6 µM. Usually 13.2 µl of primer solution is added to 186.8 µl of sterile distilled H_2O. Can be stored at -20°C for years.

Agarose electrophoresis

TBE buffer

0.5x TBE contains 44.6 mM Tris, 44.5 mM boric acid and 1 mM EDTA (pH = 8.0).

Prepare stock solution of 10x TBE. For 1 l mix 108 g of Tris, 55 g of boric acid and 40 ml of 500 mM EDTA. Undissolved white clumps may be made to dissolve by placing the bottle of solution in a hot water bath. Can be stored at room temperature for at least year.

To prepare 500 mM EDTA, add 186.15 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 with HCl; adjust volume to 1 liter with distilled water. Solution can be stored at room temperature for up to 1 year.

For 0.5x TBE dilute 10x TBE 20 times. Can be stored at room temperature for at least year.

Loading buffer

Loading buffer should contain 1% of orange G in 50% (vol/vol) glycerol. Can be stored at 4°C for years. Usually add 1.13 g of orange G to 100 ml 50% (vol/vol) glycerol.

50 bp standard

50 bp standard should contain 83.3 mg/ml 50 bp DNA ladder in 58% (vol/vol) loading buffer. For 600 µl mix 50 µl of 1 g/ml 50 bp DNA ladder, 200 µl of H_2O and 350 µl of loading buffer.

Store as 100 µl aliquotes at -20°C (can be stored for years). Store currently used aliquote at 4°C (can be used for several months).

Ethidium bromide

Stock solution should contain 1 mg/ml of Ethidium bromide (EtBr) in H_2O. Mix solution until all EtBr is dissolved (use vortex or magnetic mixer). **! CAUTION** Work with EtBr powder in fume hood, prevent inhalation. Store in the dark (cover vial with aluminium foil). Can be stored at room temperature for years.

PROCEDURE

1| DNA preparation

Prepare DNA from mouse tails or from tissues. DNA can be prepared using one of three alternative ways: Option A: Extraction by NaOH (suitable for majority of markers). In some cases you might need better quality of DNA then use more laborious Option B: DNA isolation using TRI reagent or Option C: Proteinase K method.

(A) Isolation of DNA by NaOH (based on Truett, et al. [29])

1st step ● **TIMING** ~1 min per sample + 90 min for incubation

(i) Add 600 µl of 50 mM NaOH to 1.5 ml microtube with 2 mm piece of mouse tail.

(ii) Let the tail in NaOH in 90°C for 90 min (water bath with shaking).

2nd step ● **TIMING** ~1 min per sample + 60 min for centrifugation

(i) For neutralization add 50 µl of 1M Tris pH 8.0 and vortex the samples.

(ii) Centrifuge tubes for 60 min at 3220 *g* at 4°C. Pour supernatant to new tube (0.5 ml). ■ **PAUSE POINT** DNA can can be stored in a freezer at -20 or -70 °C for years.

(iii) Concentration of DNA is approximately 40 ng/µl. For PCR dissolve DNA 1:10 in sterile distilled H_2O. If you need precise concentration of DNA measure concentration using the NanoDrop spectrophotometer.

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

(i) Homogenize 4 mm of the mouse tail or 50–100 mg of the tissue sample (fresh or frozen) with 1 ml of TRI reagent in a microtube using a Polytron 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 (vol/vol) 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 (vol/vol). 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) For PCR dilute DNA in sterile distilled water to concentration 4 ng μl^{-1}.

(C) Isolation of DNA by proteinase K

Proteinase procedure ● **TIMING** ~3 days

(i) Add 750 µl of lysis buffer, containing 100 µg ml^{-1} of proteinase K (add 5 µl of 20 mg/ml solution per 1 ml of lysis buffer) to 4 mm of the mouse tail. Lyse the samples at 55 °C overnight.

(ii) Centrifuge the samples for 60 min at 3,220 *g* at 4 °C to obtain a firm pellet.

(iii) Transmit the supernatant carefully to the microtube with isopropanol (1:1) for precipitation.

(iv) Using disposable inoculation loop withdraw precipitated DNA and transfer it into 0.5 ml of sterile distilled water. Leave DNA samples overnight at 4 °C to dissolve. ■ **PAUSE POINT** DNA can be stored in a freezer at -20 or -70 °C for years.

(vi) For PCR dilute DNA 1:10 in sterile distilled water to approx. concentration 4 ng μl^{-1}. If you need precise concentration of DNA measure concentration using the NanoDrop spectrophotometer.

2| PCR reaction

Prepare the reaction mixture. 20 µl of reaction mix should contain 0.11 µM forward and reverse primers, 0.2 mM concentration of each dNTP, 1.5 mM $MgCl_2$ (▲ **CRITICAL STEP** Optimal concentration of $MgCl_2$ for certain primers might differ), 50 mM KCl, 10 mM Tris-

HCl (pH 8.3), 0.01% gelatin, 0.4 U of REDTaq® DNA Polymerase and approximately 40 ng of sample DNA.

1st step ● **TIMING** ~15 min

Prepare PCR master mix. For different numbers of reactions see Table 1. Vortex and centrifuge PCR master mix ▲ **CRITICAL STEP** Add Taq polymerase immediately before adding mix to samples.

2nd step ● **TIMING** ~30 s per sample

(i) To each well of PCR plate put 10 µl of DNA sample (aproxx. 4 ng/µl) and add 10 µl of PCR master mix. In electrophoresis gel is distance between wells twice longer then in 96-well plate. For easier application of samples on a gel (if multichannel pipette is used) use the arrangement shown in Table 2.

(ii) Close wells by sealing tape or caps to prevent evaporation. Centrifuge the plate to flow down all reagents on the bottom of the wells.

3rd step ● **TIMING** ~2 h 45 min

Perform PCR reaction using the DNA Engine Dyad® Peltier Thermal Cycler (Bio-Rad, Hercules, CA) or similar device, 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 (▲ CRITICAL STEP optimal annealing temperature for certain primers might differ), 72°C for 60 s for elongation, and finally 3 min at 72°C for final extension. Use heated lid during PCR (100°C). ■ PAUSE POINT PCR product can be stored at -20°C for years.

3| Agarose electrophoresis

1st step ● TIMING ~2.5 h

(i) Prepare 3% agarose gel in electrophoresis plastic tray (▲CRITICAL STEP optimal gel concentration differ depending to size of PCR products analyzed: larger DNA fragments require a gel with larger pores (lower agarose percentage); smaller DNA fragments require a gel with smaller pores (higher agarose percentage)). For 3% agarose gel size 23.8 x 7.5 cm boil in microwave oven 3 g Methaphore agarose and 0.75 g UltraPure™ Agarose (this agarose is added for better mechanical properties of the gel) in 125 ml of 0.5x TBE buffer until it melts. ▲CRITICAL STEP For good dispersing add agarose to buffer, DO NOT pour buffer on agarose powder; optimal gel size may differ for certain primers. Add 7 µl of EtBr from the stock solution (1 mg/ml).

(ii) After approx. 10 min in room temperature pour gel on tray and insert comb. Let in room temperature for 1 hour. ■ PAUSE POINT Can be stored at 4°C in plastic bag for approx. one month.

(iii) Put tray into electrophoresis chamber and overlay it with 0.5x TBE buffer. Add 75 µl of EtBr stock solution (1 mg/ml), put approximately two-thirds of EtBr solution near to anode and the rest near to cathode (for electrophoresis with approximately 1.5 l of buffer).

2nd step ● **TIMING** Depends on product length and product length difference (30 min - 3 hours).

(i) Add 1 µl of loading buffer per 4 µl of PCR product solution (in Sigma REDTaq® DNA Polymerase 1U/µl solution is already enough of glycerol as well as dye and loading buffer is not necessary).

(ii) Put 10 µl of each sample solution to starting well in agarose electrophoresis (use multichannel pipette). 3 µl of standard 50 bp DNA should be loaded in the first well.

(iii) Electrophorese samples at constant voltage 150 V (▲ **CRITICAL STEP** optimal voltage may differ for certain primers), check after approximately 10 min the distance of samples from the start using Bio imaging system (Syngene) or similar device. According product size and length difference different time is needed to see the results. Usually for products length up to 100 bp it is 15-30 min, for length 100-200 bp it is 30-60 min and for length over 200 it is 45-120 min. Make photo of your result by Bio imaging system (Syngene) or similar device. ▲ **CRITICAL STEP** Make photo immediately after taking gel from electrophoresis apparatus to prevent blurring.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 3.

ANTICIPATED RESULTS

The optimized typing procedure is able to distinguish between PCR products differing in 6 bp (Figure 2). It was successfully used for mapping of genes that control response to parasites *Leishmania major*[26], *Trypanosoma brucei brucei*[27] and *Leishmania tropica*[28]. The described method is also suitable for quick test of interval-specific congenic strains, marker-assisted breeding of congenic mouse strains, test of presence of transgenes, and test of presence of knock-out or knock-in alleles in gene targeting technologies.

Whole procedure (DNA extraction, PCR reaction, electrophoresis) takes about 8.5 hours and it is possible to analyze 48 samples. When DNA is extracted in advance and prepared in master plates, the procedure (PCR reaction, electrophoresis) can be used to analyze up to 192 samples within five hours.

On agarose gel we ideally observe single band for homozygotes and two bands for heterozygotes (Figure 2). Described conditions of PCR amplification are suitable for most markers and results are readable even if non-specific bands appear. If non-specific bands overlap expected PCR product, it is necessary to optimize PCR conditions for marker primers (Table 3).

For product length difference smaller than 6 bp we use same PCR reaction but more sensitive electrophoresis system supplied by company Elchrom Scientific AG (http://www.elchrom.com/index.php?id=origins). It is also possible to use [γ-^{32}P]ATP-labeled primers and separate products in 6% acrylamide gels[30].

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TABLES

Table 1 PCR master mix. Amount of components for different number of reactions.

number of reactions*	1	6 (9)	9 (13)	12 (15)	14 (18)	18 (27)	24 (30)	24 (36)	37 (45)	36 (54)	48 (60)
Forward primer 6.7 µM	0.34	3.06	4.4	5.1	6.1	9.2	10.2	12.2	15.3	18.4	20.4
Reverse primer 6.7 µM	0.34	3.06	4.4	5.1	6.1	9.2	10.2	12.2	15.3	18.4	20.4
5x PCR buffer total	4	36	52	60	72	108	120	144	180	216	240
Sterile distilled H_2O	4.9	44.3	64.0	73.8	88.6	132.8	147.6	177.1	221.4	265.7	295.2
Taq polymerase 1U/µl	0.4	3.6	5.2	6.0	7.2	10.8	12.0	14.4	18.0	21.6	24.0
Total volume	10	90	130	150	180	270	300	360	450	540	600

units: µl

* number in brackets = theoretical number of reactions (without loss due to sticking of the liquid on the surface of tubes and pipettes)

Table 2 Positions of samples. Recommended positions of samples in plate for easier loading of samples to gel with a multichannel pipette.

	1	2	3	4	5	6	7	8	9	10	11	12
A	spl1	3	5	7	9	11	13	15	17	19	21	23
B	2	4	6	8	10	12	14	16	18	20	22	24
C	25	27	29	31	33	35	37	39	41	43	45	47
D	26	28	30	32	34	36	38	40	42	44	46	48
E	49	51	53	55	57	59	61	63	65	67	69	71
F	50	52	54	56	58	60	62	64	66	68	70	72
G	73	75	77	79	81	83	85	87	89	91	93	95
H	74	76	78	80	82	84	86	88	90	92	94	96

Table 3 Troubleshooting table.

Problem	Possible reason	Solution
There is too much product at start of electrophoresis	Too much DNA in sample	Dilute DNA sample before repeating of PCR
	Bad conditions of reaction for certain primers	Optimize reaction conditions, usually different annealing temperature or Mg^{2+} concentration is necessary
There is no product	Bad quality of DNA sample	Use DNA from stock solution or isolate new DNA
	Bad conditions of reaction for certain primers	Optimize reaction conditions, usually usually different annealing temperature or different Mg^{2+} concentration is necessary
	Bad quality of chemicals	Use new chemicals
	Contamination of solutions	Prevent contamination (use gloves, work in biohazard box etc.)
There are non-specific products overlap with PCR product	Bad conditions of reaction for certain primers.	Optimize reaction conditions, usually different annealing temperature or Mg^{2+} concentration is needed

FIGURES

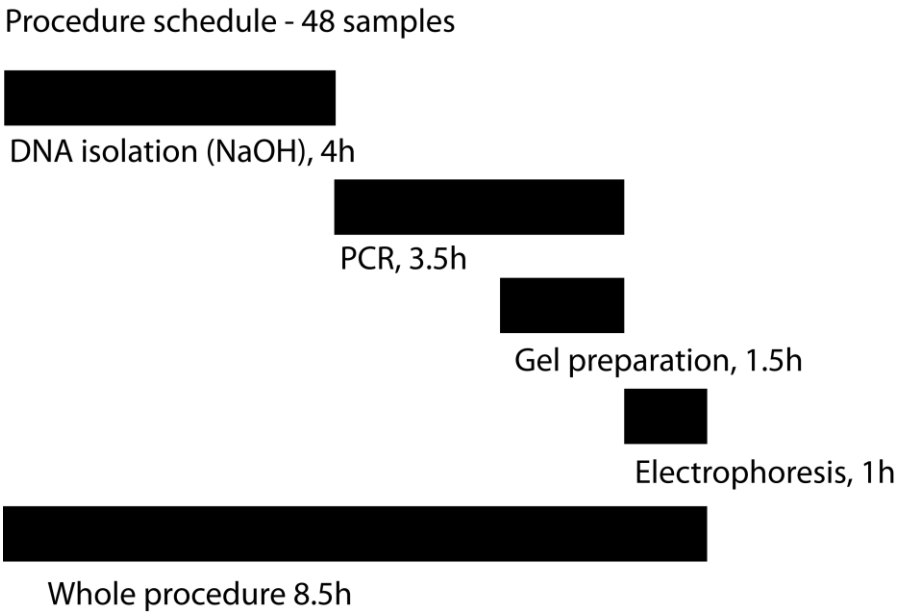

Figure 1 Time schedule of Procedure. The approximate duration of each step and the entire procedure performed with 48 samples.

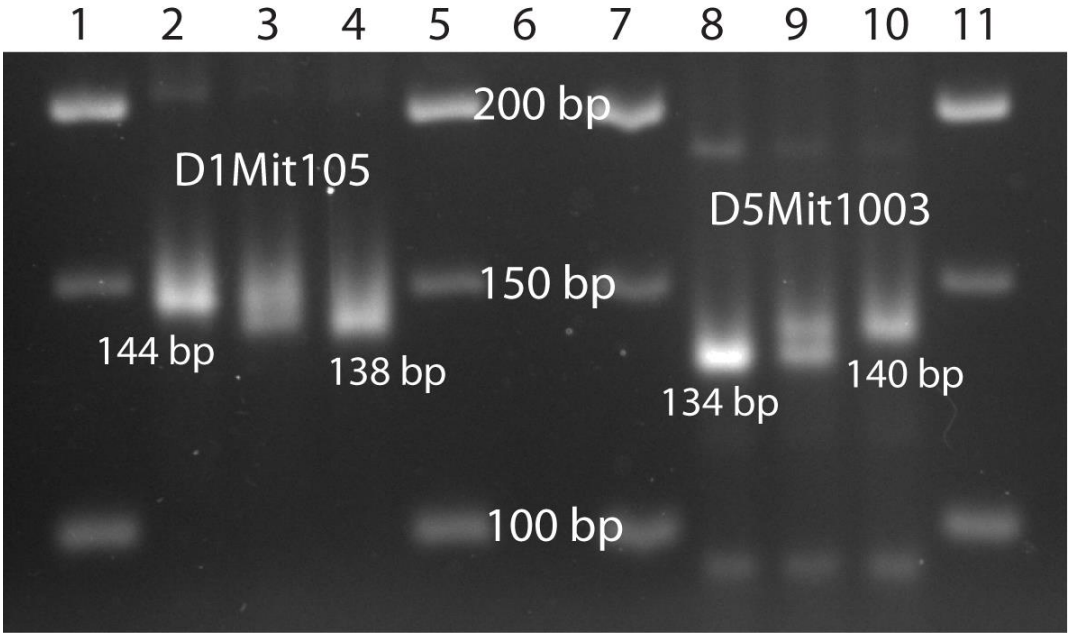

Figure 2 DNA typing using STR markers with PCR products length difference 6 bp. 1 - 50 bp ladder, 2 - BALB/c homozygote in the marker D1Mit105 (144 bp), 3 - heterozygote, 4 - STS homozygote (138 bp), 5 - 50 bp ladder, 6 - empty, 7 - 50 bp ladder, 8 - BALB/c homozygote in the marker D5Mit1003 (134 bp), 9 - heterozygote, 10 - STS homozygote (140 bp), 11 - 50 bp ladder. Gel size 23.8 x 25 cm.

5 General Discussion and Conclusions

In this thesis is presented first identification of loci controlling infections caused by *T. b. brucei* and *L. tropica* and eosinophil infiltration to inguinal lymph nodes after infection by *L. major* (Table 5). QTL mapping is a hypothesis-free approach and can lead to discovery of infection-controlling genes, which has no obvious connection with organism defensive mechanism based on current knowledge. However, it can also reveal genes with known connection to immune system playing unexpected key role in the immune response. Moreover, not only protein coding genes can affect course of the infection, but also, for example, sequences for non-coding RNAs [148].

T. b. brucei infection is controlled by 4 loci on chr. 3 (*Tbbr1*), chr. 7 (*Tbbr2*), chr. 12 (*Tbbr3*) and chr. 19 (*Tbbr4*), when *Tbbr3* and *Tbbr4* control this infection in interaction with each other. These loci were mapped in F_2 hybrids between susceptible RCS CcS-11 and parental strain BALB/cHeA. All these loci control survival after infection. We investigated discovered loci and based on current knowledge, we propose several potential candidate genes. *Tbbr1* is localized in the distal part of chromosome 3. In this site, there are present genes for two prostaglandins receptors: *Ptgfr* (prostaglandin F receptor) and *Ptger3* (prostaglandin E receptor 3 (subtypeEP3)). Prostaglandins play a suppressive role in trypanosome infection [149]. In the proximal part of chromosome 2 is situated locus *Tbbr2*, which is, due to genetic composition of the strain CcS-11, only 2.93 Mb short and contains 48 genes. There is no obvious potentially candidate between these genes, only some indirect indication about the possible role of some genes in trypanosome response, such as *Dnmt3a* (DNA methyltransferase3a) [150, 151], *Pomc* (pro-opiomelanocortin-alpha) [152, 153], *Adcy3* (adenylate cyclase 3) [154], and *Ncoa1* (nuclear receptor coactivator 1) [155]. Genes *Cd19* and *Cd5* are located at the site of *Tbbr3* and *Tbbr4,* respectively. Both codes markers of B-lymphocytes. CD19 is a B-lineage antigen [156] and B-cells plays crucial role for periodic parasitemia clearance and host survival [58]. CD-5 is marker of B-1 cells subpopulation which has been found to be stimulated by *T. cruzi* [157], *T. b. evansi* [158] and *T. congolense* [159].

In F_2 hybrids between susceptible RCS CcS-16 and parental strain BALB/cHeA we were able to map 8 loci on chr. 2 (*Ltr1*, *Ltr2*), chr. 3 (*Ltr3*), chr. 4 (*Ltr4*), chr. 10 (*Ltr5*), chr. 11 (*Ltr6*), chr. 17 (*Ltr7*) and chr. 18 (*Ltr8*). Each locus, either alone or in interaction with other locus, controls different set (from one to six) from these phenotypes: skin lesions in week 19 and 21 after infection, parasites in lymph nodes, spleen and liver, splenomegaly, hepatomegaly and serum levels of CCL3, CCL5 and CCL7. In the sites of these loci are several genes, which were previously reported to be connected to leishmaniasis. These incudes genes for cytokines and chemokines (*Il1* [160], *Il12a* [161], *Cc1* [162], *Ccl2* [163], *Ccl5* [163], *Ccl7* [164], *Ccl11* [165]), Interferonβ1 (*Ignb1* [166]), enzymes (*Hdc* [167], *Man2a1* [168], *Mmp9* [169], *Nos2* [170]), signaling and signaling pathways molecules (*Dll4* [171], *Mbd2* [172], *Mif* [173], *Ngf* [174], *Ptpn1* [175], *Smad7* [176], *Stat6* [177]), receptors (*Cd2* [178], *Cd40* [179], *Cd44* [180], *Cd74* [181], *Lgals3* [182], *Notch2* [183], *Traf6* [179], *Vcam1* [184], *Vtcn1* [185]) and transcription factor Jun (*Jun* [186]).

Eosinophil infiltration to inguinal lymph nodes after infection by *L. major* is on genetic level influenced by 4 loci on chr. 2 (*Lmr14*), chr. 5 (*Lmr25*), chr. 9 (*Lmr26*) and chr. 11 (*Lmr15*), when *Lmr15* and *Lmr26* work in interaction. These loci were mapped in F_2 hybrids between RCS CcS-9 and parental strain BALB/cHeA. In the sites of these loci are several genes that were previously described to have function in eosinophils: genes for chemokines (*Ccl3*, *Ccl5*, *Ccl7*, *Ccl11* [187], *Ccl8* [188]), proteins from BCL-2 family (*Bcl2l1* [189]), Cytokine inducible SH2-containing protein (*Cish* [190]), Guanine nucleotide binding protein (G protein), alpha inhibiting 2 (*Gnai2* [191], gene important for leukocyte diapedesis), Hemopoietic cell kinase (*Hck* [192]), High mobility group box 1 (*Hmgb1* [193], chromatin protein), interleukins (Il3 [194], Il4 [195], Il5 [196], Il13 [197]) and NOD-like receptor family, pyrin domain-containing protein 3 (Nlrp3 [198], part of inflammasome).

Table 5. Summary of loci described in this thesis.

Locus	chr.	Parasite species	Phenotype controlled
Tbbr1	3	*T.b. brucei*	survival
Tbbr2	12	*T.b. brucei*	survival
Tbbr3	7	*T.b. brucei*	survival (int. *Tbbr4**)
Tbbr4	19	*T.b. brucei*	survival (int. *Tbbr3**)
Ltr1	2	*L. tropica*	parasites in lymph nodes (int. *Ltr4**)
Ltr2	2	*L. tropica*	skin lesions week 19; skin lesions week 21; splenomegaly (int. *Ltr3**); parasites in liver; hepatomegaly; CCL7; CCL7 (int. *Ltr6**)
Ltr3	3	*L. tropica*	splenomegaly (int. *Ltr2**); parasites in spleen (transgenerational parental effect); CCL3; CCL3 (int. *Ltr7**); CCL5; CCL5 (int. *Ltr7**)
Ltr4	4	*L. tropica*	parasites in lymph nodes (int. *Ltr1**); parasites in liver (int. *Ltr8**)
Ltr5	10	*L. tropica*	splenomegaly (int. *Ltr7**); splenomegaly (int. *Ltr8**)
Ltr6	11	*L. tropica*	parasites in spleen (trangenerational parental effect); CCL7 (int. *Ltr2**)
Ltr7	17	*L. tropica*	splenomegaly (int. *Ltr5**); CCL3 (int. *Ltr3**); CCL5 (int. *Ltr3**)
Ltr8	18	*L. tropica*	splenomegaly; splenomegaly (int. *Ltr5**); parasites in liver (int. *Ltr4**); CCL7
Lmr14	2	*L. major*	eosinophil infiltration into inguinal lymph nodes
Lmr15	11	*L. major*	eosinophil infiltration into inguinal lymph nodes (int. *Lmr26**)
Lmr25	5	*L. major*	eosinophil infiltration into inguinal lymph nodes
Lmr26	9	*L. major*	eosinophil infiltration into inguinal lymph nodes (int. *Lmr15**)

* locus controls given phenotype in interaction with another locus

In all three studies we used as disease model RCS, which in phenotype parameters exceeded parameter range of both parental strains. RCS CcS-11 showed shorter survival after *T. b. brucei* infection than both parental strains BALB/cHeA and STS and other tested RCS. In previous study of *L. tropica* infection [145] female mice of RCS CcS-16 had larger lesions than parental and other tested RCS strains and unique systemic chemokine reaction, characterized by additional transient early peaks of CCL3 and CCL5, which were present neither in CcS-16 males nor in any other tested and RCS and parental strains. Also eosinophil infiltration after *L. major* infection was higher in CcS-9 strain than in other tested RCS and parental strains. This phenomenon is not rare in traits controlled by multiple genes and was well documented in other studies of immune responses of RCS [22, 24, 27, 104, 199-203] and CSS [204]. New genes combinations in RSC or CSS strain can lead to phenotypes, which are out of range of parental strains.

We observed strong sex influence on disease phenotypes. The difference in survival after *T. b. brucei* infection between strain CcS-11 and other tested strains was more prominent in females. As mention above, largest lesions and unique chemokine reaction after *L. tropica* infection were observed only in females of strain CcS-16 but not males. Eosinophil infiltration after *L. major* infection is also strongly influenced by sex. Locus *Lmr14* and two interacting loci *Lmr15* and *Lmr26* controls eosinophil infiltration only in male mice. Loci, which are sex dependent, were described before. For example, *Lmr20* influence IgE level of *L. major* infected mice only in females [92]. In *Cryptococcus neoformans* infection is pulmonary fungal burden controlled by loci *Cnes1* and *Cnes2* in female mice and by locus *Cnes3* in male mice [205]. *Chlamydia pneumonia* pulmonary infection is in mice controlled by QTLs on chromosomes 17 and 5, when chr. 17 locus has much stronger effect in males and chr, 5 locus controls this infection only in females [206]. Mouse susceptibility to influenza virus was mapped to chromosome 2 in females and to chromosome 17 in males [207]. Human genetic polymorphism (rs2069885) in gene *IL9* has opposite effect on the risk of severe respiratory syncytial virus bronchiolitis in boys and girls. Immune system of male and female mice differs [208-210]. This difference can be due to sex hormones, which modulate immune reactions [211] or it can be caused by sex-specific genetic architecture where we find profound sex-gene interactions [212] and controlling genes operates differently in both sexes.

Some discovered loci overlap with loci controlling other infections and therefore they can share the same controlling mechanisms. *Tbbr2* overlaps with locus *Lmr22* which in interaction with locus *Lmr5* controls serum level of IL-4 in *L. major* infected mice [92]. *Tbbr3* is located in the site of *Salmonella* controlling locus *Ity7* (immunity to *Salmonella typhimurium* 7) [31]. Any newly discovered *Tbbr* locus does not overlap with previous discovered loci controlling susceptibility to *T. congolense* [63, 64] or *T. cruzi* [65]. This can be caused by different regulation of immune reactions to these related parasites or by using different mouse model, which revealed different mechanisms important for the course of the disease. If we compare loci controlling *L. tropica* infection (*Ltr*) with loci

controlling *L. major* (*Lmr*) [36, 92, 108, 110, 111], we find several overlaps, which suggest same or similar mechanism of the disease control. *Ltr3* co-localizes with *Lmr11*, *Ltr5* with *Lmr5* and *Ltr8* with *Lmr13*. *Lmr14* and *Lmr15* control, among other phenotypes after *L. major* infection, the eosinophil infiltration to inguinal lymph nodes and overlap with loci *Ltr2* and *Ltr6*, respectively. The overlapping loci controlling *L. tropica* and *L. major* control different sets of phenotypes and interact with different partners, which can be caused by different function of the same gene in each infection or by coincidental co-localization of two genes, each controlling one infection. *Ltr2* and *Lmr14* are located in a site of locus *Ir2*, which controls visceral pathology after infection with *L. donovani* [109], and locus *Bb15*, which controls specific and total IgG in serum after infection with *Borrelia burgdorferi* [213]. The most probable potentially candidate gene in this site is *Il1*. IL-1 regulates visceralization in mice infected with *L. major* [160], IL-1β is upregulated in dermal lesions of patients with *L. tropica* infection [214] and *IL1B* polymorphism is connected with severity of *L. mexicana* infection [84]. Loci *Lmr15* and *Lmr26*, which control eosinophil infiltration, co-localize with loci influencing hematopoietic cell cycling [215]. Another locus controlling eosinophil infiltration, *Lmr26*, is located in the site of locus *Tria5* that modifies in vitro proliferation of mouse splenocytes stimulated by soluble anti-CD3 [23]. Summary of loci and genes controlling mouse trypanosomiasis and leihmaniasis are showed in the Figure 7.

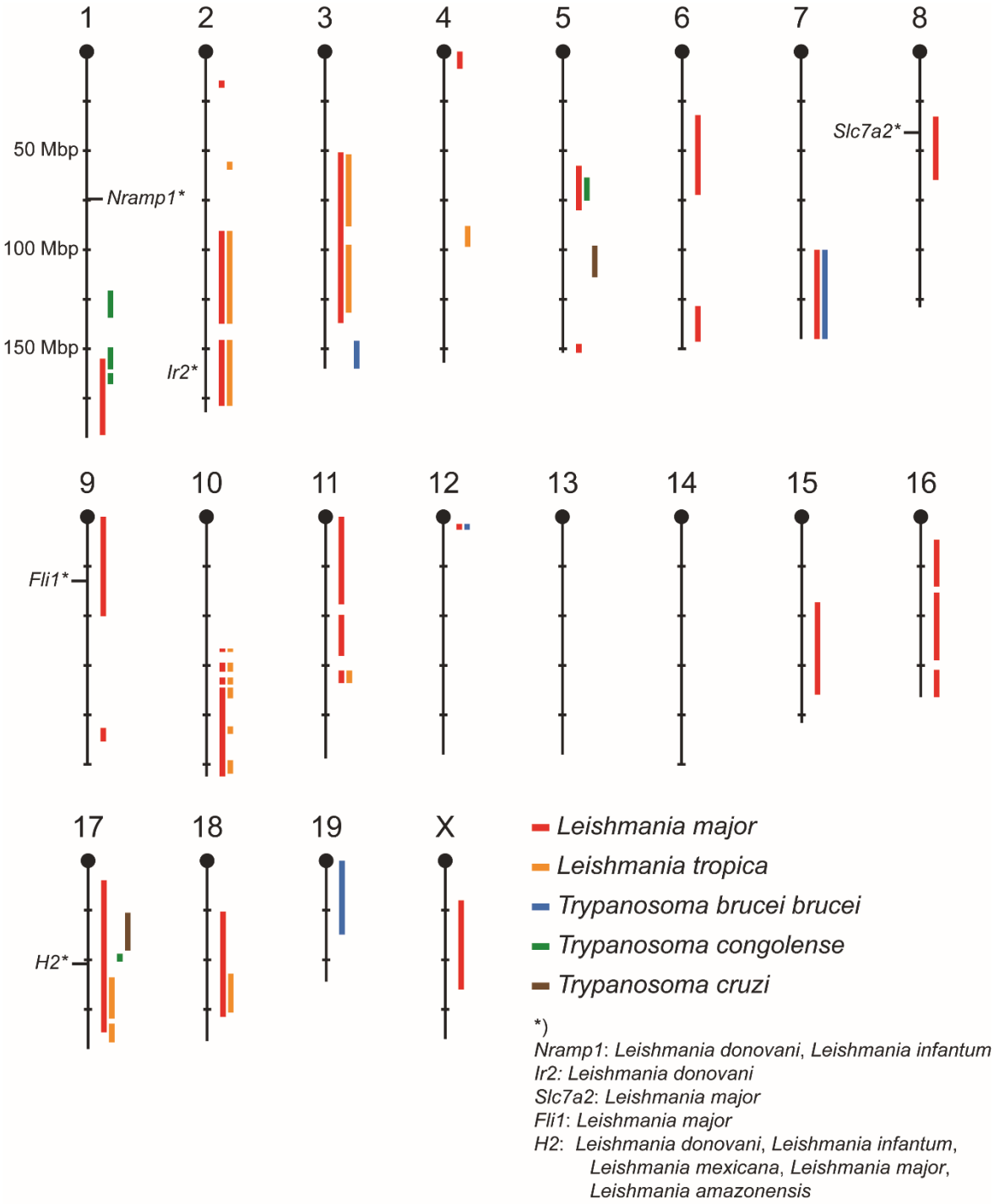

Figure 7. Mouse chromosomes with marked loci and genes that control the response to leishmaniasis and trypanosomiasis. Figure combines previously described segments with loci described in this thesis [11, 36, 63, 64, 66, 92, 94, 101, 102, 105-120].

Due to the similarity of mouse and human immune system [216] and genome homology [217] we can presume that same type of mechanism, which we find in mouse, also operate in human and this knowledge will be therefore valid for human medicine. The highest importance for future research in this field is mechanistic explanation of influence of the discovered loci/genes on disease phenotypes. One approach can be preparing of mice with very short segment from donor strain and using them in experiments to shorten controlling loci. Also analysis of genome sequence data obtained from next-generation sequencing can be very useful. Last step is functional study of potentially controlling genes.

6 References

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