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Genetic regulation of *Leishmania infection*

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Program: Molecular and Cell Biology, Genetics and Virology

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Declaration

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

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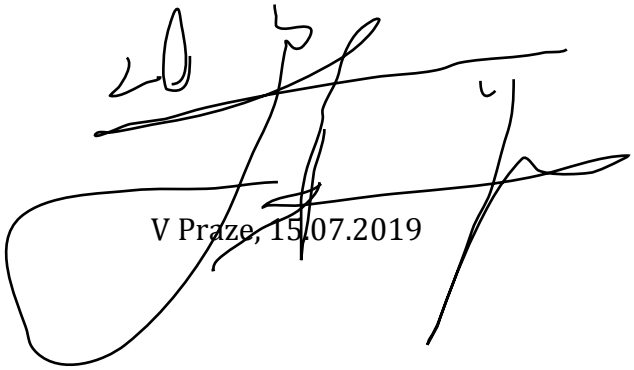

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Dedicated to

My parents

My wife Hamideh

My daughter Yasamin Zahra

My son Amir Ali

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Abstract

Leishmaniasis is a neglected tropical disease, which belongs to the top health problems because it is endemic in 98 countries in Asia, Africa, the Americas and the Mediterranean region, and is gradually expanding to new areas, including Central Europe and USA. Clinical manifestations of leishmaniasis include a diverse range of forms, ranging from non-lethal cutaneous leishmaniasis to potentially lethal visceral leishmaniasis. Asymptomatic cases are known to exist in endemic areas. Different species of *Leishmania* induce distinct symptoms, but even the patients infected by the same species develop different symptoms and may respond differently to the treatment. Thus, one of the challenges is to explain the observed variability of leishmaniasis that cannot be attributed to the currently known factors. To find novel regulatory factors of the disease we tested molecules that were shown to play role in other infections and mapped loci controlling parasite load after *L. major* infection. We also determined genetic control of survival after infection with tick-borne encephalitis virus (TBEV) in order to establish whether there are common elements in response to *L. major* and TBEV.

Interferon-induced GTPases (guanylate-binding proteins, GBPs) play an important role in inflammasome activation and mediate innate resistance to many intracellular pathogens, but little is known about their role in leishmaniasis. Fcγ receptor IV (FCGR4), the receptor for the Fc fragment of immunoglobulin G (IgG), participates in IgG2a- and IgG2b-dependent effector functions in immune response. Experiments with mice bearing knockouts of other Fcγ receptors have shown that the genetic background of the host controls their role in response to *Leishmania* parasites, leading either to protective

immunity or to progression of disease. However, the information about the role of FCGR4 in leishmaniasis is missing.

We therefore studied expression of *Gbp2b*/*Gbp1*, *Gbp5* and *Fcgr4* mRNA in skin, inguinal lymph nodes, spleen and liver after *L. major* infection and in uninfected controls. We used two different groups of related mouse strains, which were classified on basis of size of infection-induced skin lesions as highly susceptible (BALB/c, CcS-16), susceptible (B10.O20), intermediate (CcS-20), and resistant (STS, O20, B10, OcB-9, OcB-43).

We observed strong genetic influence on *Gbp2b*/*Gbp1* and *Gbp5* mRNA levels. Some strains differed in *Gbp2b*/*Gbp1* and *Gbp5* expression prior to infection. Several of them differed in *Gbp2b*/*Gbp1* and/or *Gbp5* expression although they carried the same *Gbp2b*/*Gbp1* and/or *Gbp5* alleles, indicating their trans-regulation.

Infection resulted in approximately 10x upregulation of *Gbp2b*/*Gbp1* and *Gbp5* mRNAs in organs of both susceptible and resistant strains. It was most pronounced in skin. Co-localization of GBP2b protein with most *L. major* parasites in skin of resistant and intermediate strains, but not in highly susceptible BALB/c mice suggests that this molecule might play role in defense against leishmaniasis.

Similarly as in study of *Gbps* expression, we observed strong genetic influence on *Fcgr4* mRNA levels, as well as trans-regulation in both uninfected and infected mice. Infection caused a varying degree of up-regulation (up to 50x) of *Fcgr4* in organs of mouse strains irrespective of their susceptibility or resistance. The up-regulation of FCGR4 after infection was confirmed by immunohistochemistry. We have compared localization of the parasites and FCGR4 in skin and liver of selected strains and found their partial co-

localization. These findings suggest relationship of this molecule to the response to *L. major* infection.

The development of visceral leishmaniasis, which is lethal if untreated, is not yet understood. Therefore we analyzed the genetics of parasite load, spread to internal organs, and ensuing visceral pathology. Quantification of *Leishmania* parasites in lymph nodes, spleen and liver of infected F_2 hybrids between BALB/c and recombinant congenic strains CcS-9 and CcS-16 allowed us to describe a network-like set of interacting genetic loci that control parasite load in different organs. We mapped two novel loci controlling parasite load: *L. major* response 24 and 27 (*Lmr24* and *Lmr27*). We also detected parasite-controlling role of the previously described loci *Lmr4*, *Lmr11*, *Lmr13*, *Lmr14*, *Lmr15* and *Lmr25*, and describe 8 genetic interactions between them. *Lmr14*, *Lmr15*, *Lmr25* and *Lmr27* controlled parasite load in liver and lymph nodes. In addition, *Leishmania* burden in lymph nodes but not liver was influenced by *Lmr4* and *Lmr24*. In spleen, parasite load was controlled by *Lmr11* and *Lmr13*. We detected a strong effect of sex on some of these genes.

In the last part of presented thesis we explored genetic control of susceptibility to TBEV. Our previous data shown that the strain CcS-11 carrying 12.5% of the STS genome on the background of the genome of the strain BALB/c, is even more susceptible than parental strains. Therefore, we have generated a F_2 intercross between BALB/c and CcS-11 and performed a linkage and bioinformatics analysis. These studies revealed a novel suggestive locus on mouse chromosome 7 containing 9 potential candidate genes for TBEV response. Interestingly, on the mouse chromosome 7 was in the strain CcS-11 mapped locus *Lmr21* that controls susceptibility to *L. major*. However, this locus is mapped on a long chromosomal segment, thus other gene(s) might be responsible for its effect.

Collectively, the thesis presents the new insight on the response to *L. major* parasites. For the first time, we described the role of *Gbps* and *Fcgr4* as inflammatory markers of *L. major* infection. Our results also point out that expression of *Gbps* and *Fcgr4* was increased even in organs of resistant mice, which might suggest a hidden inflammation. It remains to be established whether the clinically asymptomatic infection might represent danger in predisposing organism to other diseases. We also provided a first systematic a genome wide search of the genetic control of parasite load in mammalian organs after *L. major* infection. Host genes controlling *L. major* revealed a wide variety of heterogeneous effects that included distinct organ-specific control, single-gene effects, gene-gene interactions and sex dependent control.

Further deep characterization of role of *Gbps*, *Fcgr4* genes and fine mapping of newly identified loci involved in controlling parasite burden in different organs after *Leishmania* infection may help to understand the detailed mechanisms of the disease and would open new perspectives of the research, treatment and vaccine development against leishmaniasis.

Abstrakt

Leismanióza je opomíjené tropické onemocnění, které se řadí k nejzávažnějším zdravotnickým problémům. Je endemické v 98 zemích Asie, Afriky, Ameriky a Středomoří a postupně se šíří do nových oblastí včetně střední Evropy a USA. Klinické formy leishmaniózy zahrnují široké spektrum projevů od kožní leishmaniózy až po potenciálně smrtelnou viscerální leishmaniózu. V endemických oblastech je znám výskyt asymptomatických případů. Různé druhy leishmanií indukují různé symptomy, ale dokonce pacienti infikovaní stejným druhem parazita mohou projevovat různé symptomy onemocnění a různě reagovat na léčbu. Proto je jednou z velkých výzev vysvětlení pozorované variability, kterou nelze vysvětlit pomocí současně známých faktorů. Abychom našli nové faktory regulující leishmaniózu, testovali jsme úlohu molekul, o nichž je známo, že se účastní regulace jiných infekcí, a mapovali jsme lokusy kontrolující množství parazitů po infekci *L. major*. Také jsme determinovali kontrolu přežití po infekci virem klíšťové encefalitidy (TBEV) s cílem určit, zda existují společné prvky v odpovědi k *L. major* a TBEV.

Interferonem indukované GTPázy (guanylate-binding proteins, GBPs) hrají významnou úlohu v aktivaci inflamazómu a zprostředkovávají vrozenou rezistenci proti mnoha vnitrobuněčným patogenům. O jejich roli v leishmanióze je ale známo jen velmi málo. Fcγ receptor IV (FCGR4), receptor pro Fc fragment imunoglobulinu G (IgG), se podílí na efektorových funkcích imunitní odpovědi závislých na IgG2a a IgG2b. Pokusy s myšmi, které měly inaktivované jiné Fcγ receptory, ukázaly, že genetické pozadí hostitele určovalo jejich úlohu v odpovědi k parazitům a vedlo buď k rozvoji pretektivní imunity nebo k zhoršení projevů nemoci. Úloha FCGR4 v leishmanióze není známa.

Proto jsme studovali expresi mRNA pro *Gbp2b/Gbp1*, *Gbp5* a *Fcgr4* v kůži, ingvinálních lymfatických uzlinách, slezině a játrech po infekci *L. major* a u neinfikovaných kontrol. Použili jsme dvě různé skupiny příbuzných myších kmenů, které byly klasifikovány na základě velikosti kožních lezí způsobených infekcí jako vysoce vnímavé (BALB/c, CcS-16), vnímavé (B10.O20), středně odolné (CcS-20) a rezistentní (STS, O20, B10, OcB-9, OcB-43).

Pozorovali jsme silné genetické ovlivnění hladin mRNA *Gbp2b/Gbp1* a *Gbp5.* Některé kmeny se lišily v expresi *Gbp2b/Gbp1* a *Gbp5* již před infekcí. Několik z nich se odlišovalo v expresi *Gbp2b/Gbp1* a/nebo *Gbp5*, přesto, že nesly stejné alely *Gbp2b/Gbp1* a/nebo *Gbp5,* což odhalilo trans-regulaci *Gbps*.

Infekce vedla k přibližně desetinásobné upregulaci mRNA *Gbp2b/Gbp1* a *Gbp5* v orgánech vnímavých i rezistentních kmenů a byla nejvíce výrazná v kůži. Ko-lokalizace proteinů GBP2b a parazitů *L. major* v kůži odolných a středně odolných kmenů, ale ne v kůži vysoce vnímavého kmene BALB/c naznačila, že tato molekula může mít úlohu v obraně proti leishmanióze.

Podobně jako ve studii exprese *Gbps* jsme pozorovali silné genetické ovlivnění hladin mRNA *Fcgr4* a také trans-regulaci exprese jak u neinfikovaných, tak u infikovaných myší. Infekce způsobuje v orgánech myších kmenů různou míru upregulace (až 50x) genu *Fcgr4* nazávisle na vnímavosti nebo rezistenci. Upregulace FCGR4 po infekci byla potvrzena immunohistochemicky. Srovnali jsme umístění parazitů a FCGR4 a zjistili jejich částečnou ko-lokalizaci. Tento nález naznačuje vztah této molekuly a odpovědi k infekci *L. major*.

Jak dochází k rozvoji viscerální leishmaniózy, která je bez léčby smrtelná, není dosud známo. Proto jsme analyzovali genetické ovlivnění počtu parazitů, jejich šíření do

vnitřních orgánů a následnou viscerální patologii. Určení počtu parazitů v lymfatických uzlinách, slezině a játrech infikovaných F_2 hybridů mezi kmenem BALB/c a rekombinantními kongenními kmeny CcS-9 a CcS-16 nám umožnilo popsat síti podobný soubor interagujících genetických lokusů, které kontrolují množství parazitů v různých orgánech. Zmapovali jsme dva nové lokusy, které determinují množství parazitů: *Leishmania major* response 24 a 27 (*Lmr24* a *Lmr27*). Také jsme detekovali roli v kontrole parazita u dříve popsaných lokusů *Lmr4*, *Lmr11*, *Lmr13*, *Lmr14*, *Lmr15* and *Lmr25* a *Lmr27* a popsali 8 genetických interakcí mezi nimi. *Lmr4*, *Lmr15*, *Lmr25* a *Lmr27* kontrolují množství parazitů v játrech a lymfatických uzlinách. Dále jsme zjistili, že množství leishmanií v lymfatických uzlinách, ale ne v játrech je ovlivněno *Lmr4* a *Lmr24*. Ve slezinách bylo množství parazitů kontrolováno *Lmr11* a *Lmr13*. Detekovali jsme silný vliv sexu na některé z popsaných genů.

V poslední části předkládané práce jsme studovali genetickou kontrolu vnímavosti k TBEV (virus klíšťové encefalitidy). Naše předchozí výsledky ukázaly, že kmen CcS-11, který nese 12,5% STS genomu na pozadí genomu kmene BALB/c, vykazuje vyšší vnímavost než rodičovské kmeny. Proto jsme přípravili F_2 kříženec mezi kmeny BALB/c a CcS-11 a provedli vazebnou a bioinformatickou analýzu. Tyto studie odhalily nový pravděpodobný lokus na myším chromozomu 7, který obsahuje 9 potenciálních kandidátních genů pro vnímavost k TBEV. Je zajímavé, že na myším chromozomu 7 byl u kmene CcS-11 mapován lokus *Lmr21*, který kontroluje vnímavost k *L. major*. Tento lokus je však zmapován na dlouhém chromozomálním úseku, takže za popsané účinky mohou být zodpovědné různé geny.

V souhrnu práce prezentuje nový vhled do odpovědi k parazitu *L. major*. Jako první popisuje roli genů *Gbps* a *Fcgr4* jako markerů zánětu způsobeného infekcí *L. major*. Naše výsledky také ukazují, že exprese *Gbps* a *Fcgr4* byla zvýšena dokonce v orgánech rezistentních myší, což může naznačovat skrytý zánět. Zbývá určit, zda klinicky asymptomatická infekce může představovat nebezpečí tím, že organizmus disponuje k náchylnosti k dalším chorobám. Také jsme provedli první systematický celogenomový screening pro odhalení genů kontrolujících množství parazitů v savčích orgánech po infekci *L. major*. Ukázalo se, že tyto geny vnímavosti hostitele se vyznačují heterogenními účinky zahrnujícími orgánově-specifickou kontrolu, geny se samostatným účinkem, genové interakce a geny, jejichž vliv je vázaný na pohlaví.

Další hlubší charakterizace role genů *Gbps* a *Fcgr4* a přesné mapování nově identifikovaných lokusů podílejících se na kontrole množství parazitů po infekci leishmanií v různých orgánech může pomoci porozumět podrobným mechanismům nemoci a otevřít nové perspektivy výzkumu, léčby a vývoje vakcíny proti leishmanióze.

Contents

Chapter 1; Introduction

Leishmaniasis

Leishmaniasis is a neglected tropical disease which is caused by a group of vector-borne parasites and is endemic in 98 countries on 5 continents (Figure 1), causing 20,000 to 40,000 deaths per year [1].

The disease is transmitted by female sand flies of the genera *Lutzomyia* and *Phlebotomus*, and is caused by 20 different species of protozoan parasites belonging to the genus *Leishmania*, although the taxonomic status of some of them is disputed.

It has been reported that more than 350 million people are at risk. However, due to the prevalence of leishmaniasis in socio-economical areas that are considered as developing or under-developed countries, consistency surveillance and reporting has been compulsory in only 32 of the 98 countries estimated incidence seems to be much higher. These facts, accompanied with high cost of medical treatment, the real numbers of infected individuals might be much higher [1; 2]. The number of cases reported globally has increased over the past 10 years in part due to improved diagnostics, but also due to increasing numbers of drug resistant cases, and a lack of adequate vector or reservoir control tools [3]. Ecological changes like urbanisation, deforestation and climate changes as well as economic crises, natural disasters, armed conflicts and tourism to endemic areas result in spreading or exposure of susceptible populations to leishmaniasis and increasing number of affected cases [3]. Leishmaniasis is a global health challenge, as there is no safe and effective vaccine against any form of human leishmaniasis. Chemotherapy either have severe side effects [4] or its use in developing countries is limited due to the high market prices [5]. The emergence of HIV/AIDS infection and co-infection of HIV/*Leishmania* is

another global health challenge, leading to modifications on the distribution map of leishmaniasis [6; 7].

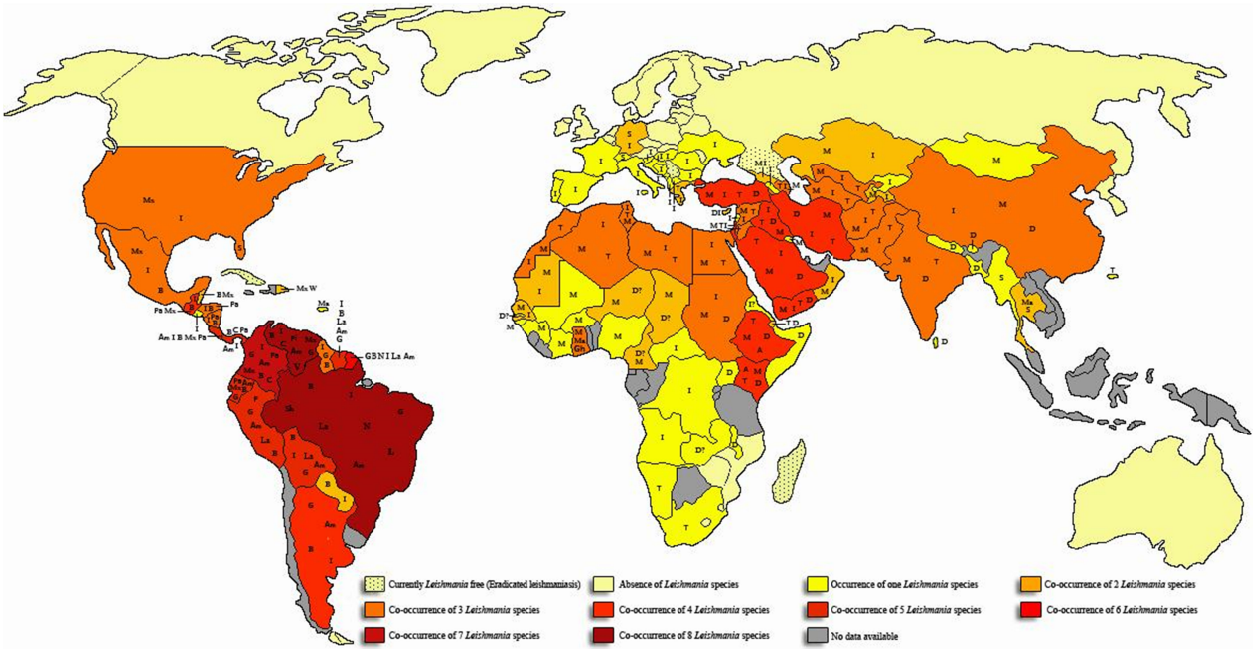

Figure 1. Distribution of the 20 Leishmania species pathogenic for humans. The species name has been shown as abbreviation. A: *L. aethiopica*; Am: *L. amazonensis*; B: *L. braziliensis*; C: *L. colombiensis*; D: *L. donovani*; G: *L. guyanensis*; Gh: *'Ghana strain'*; I: *L. infantum*; La: *L. lainsoni*; L: *L. lindenbergi*; M: *L. major*; Ma: *L. martiniquensis*; Mx: *L. mexicana*; N: *L. naiffi*; Pa: *L. panamensis*; P: *L. peruviana*; S: *L. 'siamensis'*; Sh: *L. shawi*; T: *L. tropica*; V: *L. venezuelensis* and W: *L. waltoni*. The species with question marks need to be confirmed by further genotyping studies [8].

Treatment of leishmaniasis is difficult because of the lack of reliable drugs. Existing leishmanicidal agents show severe side effects. In addition, the treatment is costly and not readily available to a majority of patients. In spite of numerous attempts to develop vaccination against leishmaniasis, there are still no safe and effective vaccines suitable for humans [4]. Clinical form and susceptibility to leishmaniasis are dependent on parasite

species, environmental and social factors, and also on nutrition and genotype of the host [9; 10; 11].

Clinical form of leishmaniasis includes a diverse range of forms, ranging from non-lethal cutaneous leishmaniasis (CL) to potentially lethal visceral leishmaniasis (VL).

Asymptomatic cases are known to exist in endemic areas, and may act as an important reservoir for re-infection[12]. The reservoir hosts of most *Leishmania* species from which parasites are transmitted to humans are rodents and canids (zoonotic leishmaniasis). only in some species (for example, *L. tropica* in urban areas) transmission from is infected to non-infected humans (anthroponotic leishmaniasis) [9]. The parasite has minimally **two** stages of life cycle in at least two hosts; sandfly vector and vertebrate (Figure 2).

Life cycle of *Leishmaina* starts with injecting a small number of infectious metacyclic promastigotes by a female sandfly into the skin. These forms are efficiently taken up by myeloid derived phagocytic cells mainly macrophages [MΦ] and dendritic cells [DC] however they can infect also neutrophils, and fibroblasts of the mammalian host [9; 13; 14]. Upon phagocytosis *Leishmania* survive and multiply inside the macrophages. These parasitized cells can be taken up again by another sanfly during a blood meal (Figure 2) [9].

The parasites transform into non-flagellated amastigotes forms. Infected cells are taken up by another sandfly during blood meals and lysed in the midgut where it transforms into promastigotes (procyclic promastigotes). These forms attach to the midgut wall and differentiate into non-dividing metacyclic promastigotes, which can be transmitted into a mammalian reservoir or a host depending the species of the parasite[15].

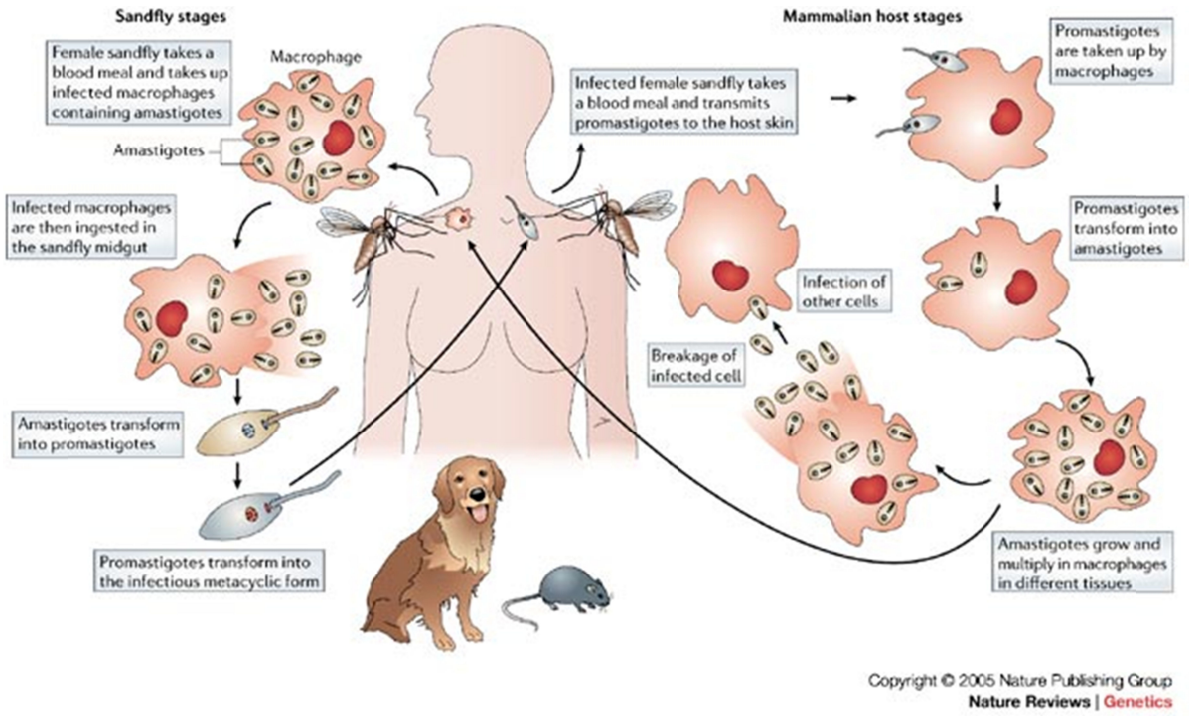

Figure 2. Infection cycle of leishmaniasis [9]

Clinical forms of leishmaniasis

The course of the disease associated with *Leishmania* infections is primarily dependent upon the parasite species, but also may be determined in part by the host genetic background, the inoculation size, nutritional status of the host [9; 16; 17; 18; 19] . In addition, environmental and social factors also have a considerable impact on the outcome of the disease [3; 9]. In humans, different species of *Leishmania* parasite can give rise to distinct clinical manifestations, ranging from a self-healing singular or multiple skin ulcers in cutaneous leishmaniasis to systemic disease in visceral leishmaniasis. Clinical

symptoms of leishmaniasis can be found in three main clinical pictures: cutaneous, mucocutaneous and visceral leishmaniasis (Figure 3) [3].

Figure 3. Clinical forms of leishmaniasis, from the left, CL, MCL and VL

Cutaneous leishmaniasis (CL)

CL is the most common and widely distributed form of leishmaniasis is characterized by papules and nodules which ulcerates and crusts over (Figure 3A). The lesion is usually localized to the site of sandfly bite on exposed parts of the body, more often on face, hands or feet. CL is present in the Middle East, in Central Asia and South America; Mediterranean region of Europe and the North and Central Africa. It is mainly caused by *L. amazonensis, L. braziliensis, L. guyanensis, L. mexicana, L. panamensis, L. naiffi, L. venezuelensis, L. lainsoni and L. shawi* in the New World and *L. major, L. aethiopica, L. tropica, L. Arabica* and *L. gerbilli* in the Old World. However, other species such as *L. donovani* and *L. infantum* can also cause the disease [20]. Although most of the cases of CL

are self-healing within several months or 1-2 years after infection but, they rarely convert to non-healing form refractory to all types of remedies.

Mucocutaneous leishmaniasis (MCL)

Mucocutaneous leishmaniasis (MCL) is the most feared form of leishmaniasis (Figure 3B). MCL it is very much common in Latin America but it can be seen also in African countries like in Ethiopia and Kenya. The disease results after dissemination of the parasites from the site of infection into mucosal areas of the nasopharyngeal region nose, mouth and throat cavity and surrounding tissues. MCL is mostly caused by *L. braziliensis*. but other species may also cause the disease, such as *L. panamensis and L. guyanensis or L. aethiopica* [20].

Visceral leishmaniasis

VL is the most severe form of the disease with very high mortality rate if not treated. The parasite invades the phagocytic cells of reticuloendothelial system, particularly the liver, spleen, bone marrow, lymph nodes and intestines. Hepatosplenomegaly, splenomegaly and severe immunodeficiency are hallmarks of VL (Figure 3C). VL is caused by *L. donovani, L. chagasi* and *L. infantum* occur mainly in poor rural and suburban areas of 5 countries: Bangladesh, India, Nepal, Sudan and Brazil [21; 22; 23]. However, less common VL also may occur after *L. tropica* infection. Hepatosplenomegaly, splenomegaly and severe immunodeficiency are hallmarks of VL. Post-Kala-azar Dermal Leishmaniasis (PKDL) is a complicated form of VL which occurs within months or a year after VL if the case remains untreated or incompletely treated.

The disease is characterized by a nodular rash, hypopigmented macules and/or butterfly erythema of the face [21; 22].

Immune response against leishmanaisis

It is accepted now that a Th1 cell-mediated type of response is appropriate for combating infection by intracellular pathogens (e.g. *Leishmania* parasites), whereas Th2 response is required for protection against extracellular pathogens [15]. Extensive study on the murine model of leishmaniasis caused by *L. major* showed that the general mechanism of the protective immune response against *Leishmania* infection is held via activation of multifunctional CD4+ T cells, boosting production of IFN-γ, IL-2, and TNF and limiting levels of IL-4 and IL-10 [24]. Ultimately, Th1-cytokine producing cells overwhelm IL-4 producing cells, and upregulate antimicrobial effector mechanisms, including the acidification of the phagosomes, expression of iNOS and subsequent generation of nitric oxide by infected macrophages and neutrophils which results in killing the parasite and resolving the infection [9; 15; 25]. IL-12 stimulates phagocyte-dependent immunity and promotes development of cellular immunity and a robust Th1 response against intracellular pathogen and *Leishmania* infection [26]. The most important effector cytokines in the Th1 response are IFN-γ and TNF-α, which are the key molecules in activation of macrophages in classical way [27]. At the very early stage of the infection, IFN-γ is mainly supplied by natural killer (NK) cells [28; 29]. There are also evidences that myeloid-derived suppressor cells (MDSCs) are also involved in susceptibility of leishmaniasis. During experimental leishmaniasis, resistant C57BL/6 mice accumulated MDSCs which suppressed parasite-specific T-cell proliferation, but not cytokine release

[30]. In agreement with these observations, genetically susceptible BALB/c mice exhibited a reduced ability to recruit CD11b+ Ly6Chi cells to the site of *L. major* infection compared to resistant C57BL/6 mice. Thus, genetic conditioning of MDSC function could contribute to disease resistance in murine inbred strains [30].

In spite of protective immune response in a resistant host, the early production of IL-4 and/or IL-13 is associated with susceptibility to leishmaniasis [31]. Nevertheless it was shown that when present during the initial activation of dendritic cells (DCs) by infectious agents, IL-4 instructed DCs to produce IL-12 and promote TH1 development [32]. It has been identified that oligoclonal population of T cells possessing a Vβ4Vα8 T cell receptor are the main supplier of early IL-4 which was produced in response to the *Leishmania* homolog of receptors for activated kinase (LACK) antigen [33]. IL-13 and regulatory cytokine IL-10 are also important in development of Th2 response in susceptible mice [34; 35]. These molecules not only promote Th2 response but also down-regulation of IL-12 receptor and eventually Th1 response and increased inflammatory cell infiltrate [15; 35]. Interestingly it was shown that production of IL-4 or IL-10 enhances CD206 expression which favors uptake of *Leishmania major* Seidman which develops non-healing cutaneous lesion[36].

It is now well appreciated that the mechanisms involved in the immunopathology of leishmaniasis are very complex and despite an important role of Th1/Th2 cells in creation of resistance/susceptibility to *Leishmania* infection, it seems that many other cell types, and immunological and physiological factors are involved in final outcome of the disease. Recent investigations have added further complexity to the parameters, which are involved

in mechanisms of the disease. It was shown that the Th17 CD4 cells which are present in high number in infected BALB/c mice are associated with susceptibility to *L. major* parasites, [37; 38]. Defect in IL-17 production in BALB/c mice is associated with resistance to *L. major* [37]. It was also shown that Th9 cells and T follicular helper cells (Tfh) could also be involved in susceptibility to leishmaniasis [38]. Increasing neutrophil recruitment may also promote susceptibility to *Leishmania* [37; 38]. However, Th1/Th2 dichotomy in the human system is not as distinct as in mice. Studies in *L. major* and *L. braziliensis* infection demonstrated a mixed Th1/Th2 response in early stage of active CL [11], [42] and then a sustained Th1 response with elevated level of IFN-γ and down-regulation of IL-4 and IL-10 production were seen apparently associated with healing [11]. Nowadays, there are appropriate amount of evidence showing how complex immune responses to leishmaniasis are and many additional factors are involved.

In humans, most of the evidence suggesting the association of Th1 with cure and immunity is applicable to CL. In contrast, in chronic CL or ML cases, a combination of Th1 and Th2 response is observed [3]. In VL, a different pattern of cytokine profile occurs [38; 39]. Activation of Th1, Th2, Th17 or other cell types, along with cytokines and chemokines production and infiltration of effector cells into the site of infection, orchestrates the corresponding type of immune response against the diseases [38]. Appearance of clinical symptoms and immune responses in leishmaniasis are strongly dependent on *Leishmania* species and presence of parasitic factors which are involved in virulence of the parasite[36].

Guanylate Binding Proteins (GBPs)

Upon infection and inflammation, antigen presenting cells produce IL12 and IL18 which initiate local IFNγ production in NK cells. Production IFNγ attract TH1 and cytotoxic T cells which leads to increasing amounts of IFNγ locally[40]. In addition, TH17, TH2 and TFH, ILC subsets have also been reported to release IFNγ under specific conditions at the site of inflammation [40; 41]. Inducible GTPases are the most abundantly expressed proteins after IFNγ stimulation [42]. The GBPs were amongst the first described inducible GTPases[42]. Guanylate-binding proteins (GBPs) are components of cell-autonomous immunity playing a key role in response to intracellular infections, regulation of inflammasomes—a high-molecular-weight complexes present in the cytosol of stimulated immune cells that mediate the activation of inflammatory caspases resulting in pathogen clearance and/or death of infected cell [43; 44; 45; 46]. Besides their role in defense against pathogens, they influence cellular proliferation, adhesion, and migration [reviewed in Ref.[44]], and some members have direct anti-tumorigenic effect on tumor cells [47]. A wide range of studies revealed an important role of GBPs in response to different infections including viral [48; 49; 50; 51], bacterial [52; 53], and protozoan pathogens [54; 55; 56], both vacuolar [55; 56; 57] and cytosolic [48; 49; 58]. Gbps can also attack parasites directly via supramolecular complexes [59] and interfere with virus replication [48] or virion assembly [49]. Type of effective defense depends on pathogen involved.

GBPs and Gbps were first detected as a 67 kDa protein fraction after stimulation of different human and mouse cell lines with IFN [60] and further characterized as a GBP after stimulation of human and mouse fibroblasts with IFNα, IFNβ, and IFNγ[61]. There are currently seven GBPs known in humans (encoded by genes located on the chromosome 1)

[reviewed in Ref. [43; 62]] and 11 Gbps in mouse. Gbp2b/Gbp1, Gbp2, Gbp3, Gbp5, and Gbp7 map to chromosome 3, whereas Gbp4, Gbp6, Gbp8, Gbp9, Gbp10, and Gbp11 are localized on chromosome 5 [63]. These proteins are highly conserved and belong to dynamin superfamily—multidomain mechano-chemical GTPases, which are implicated in nucleotide-dependent membrane remodeling events [64; 65].

Immune cells such as monocytes [55] increase expression of GBPs during infections like Slmonella, after stimulation with IFNγ, IFNβ and/or stimulation with IL1β, LPS or CpG oligodeoxynucleotide [55]. The level of induction is very much depends on the stimulant.

Murine Gbp2b/Gbp1 plays role in defense against *Listeria monocytogenes* and *Mycobacterium bovis* BCG [53], Gbp2 inhibits replication of vesicular stomatitis virus and encephalomyelocarditis virus [58], *Toxoplasma gondii* [66] and *Salmonella typhimurium* [57], Gbp5 protects against *Salmonella typhimurium* [52] and *Mycobacterium bovis* BCG [53]. Moreover, several Gbps can cooperate for more effective defense. Gene specific-silencing using siRNA established that murine Gbp2b/Gbp1, Gbp5, Gbp7 and Gbp6/10 protect against *M. bovis* BCG and *L. monocytogenes.* A combination of siRNAs exacerbated the loss of function, which indicated that protective Gbps functioned cooperatively [53]. Similarly, mutual molecular interactions of murine Gbp2b/Gbp1, Gbp2, Gbp3, Gbp5 and Gbp6 protected against *T. gondii* [59].

There is very little known about a possible role of GBPs in *Leishmania* infection. Analysis of global gene expression of bone marrow derived macrophages from BALB/c mouse demonstrated upregulation of expression of Gbp2b/Gbp1, Gbp2, Gbp3, Gbp6, and Gbp7 after 24 hours of infection with *L. major* promastigotes [67]. Dendritic cells generated from blood of healthy human donors exhibited increased expression of GBP1 and GBP2

16 hours after infection by *L. major* promastigotes, whereas dendritic cells infected by *L. donovani* had increased expression of GBP1 [68]. To extend knowledge about role of Gbps after *Leishmania* infection, we studies expression of Gbp1/2/and Gbp5 in susceptible and resistant mice.

Fcγ receptor IV

IgG antibodies participate in defense of the organism against pathogens. They can neutralize infectious agents and their products by inhibiting a part of a pathogen that is essential for its invasion and survival, by activating classical complement pathway and by binding to Fcγ receptors (FcγRs) on the membrane of immune cells. Depending on the cell and FcγR type, crosslinking of FcγRs on the cell surface activates several effector functions including phagocytosis, stimulation of respiratory burst, cell degranulation, antibody-dependent-cell-mediated cytotoxicity, and expression of chemokines and cytokines [69; 70]. Depending on their functions, there are two main groups of the FcγRs: activating and inhibitory receptors. Activating FcγRs either have an immunoreceptor tyrosine-based activation (ITAM) motif in their intracytoplasmic domain or associate with the ITAM-containing signaling subunit FcR common γ chain, while inhibitory receptors carry an immunoreceptor tyrosine-based inhibition (ITIM) motif. FcγRs are also classified depending on their affinity for IgGs. A few receptors can bind to monomeric IgG, which is definition of high-affinity receptors, while the other receptors predominantly bind to aggregated IgGs [71].

Mouse Fcγ receptor IV (FCGR4) is a glycoprotein that consists from the α chain with two extracellular globular domains and the dimer of the FcR common γ chain and its

expression is restricted to myeloid-derived cell lineages: monocytes/macrophages, neutrophils and monocyte-derived dendritic cells [71; 72; 73]. FcγRIV is encoded by the gene Fcgr4 (also called Fcgr3a, Fcrl3 and CD16-2) that is situated on the distal part (171.02 Mbp; 78.53 cM) of the mouse chromosome 1 in the Fcgr cluster between the genes Fcgr2b and Fcgr3 [74]. FcγRIV bounds to the mouse IgG2a and IgG2b isotypes [72; 75] and participates in IgG2a- and IgG2b-dependent effector functions in autoimmune responses and antibody-dependent cell-mediated cytotoxicity. FcγRIV can also interact with IgE, which is involved in phagocytosis and inflammatory response by macrophages [76]. This activation is restricted to mouse strains that carry IgEb allele of the heavy chain of IgE (Igh-7) [76] present in the strains C57BL/6, SJL/J, C57BL/10A, but not in the strains BALB/c, C3H/HeN, C3H.SW/Hz, CBA/J, A/J and AKR/J [77]. Fcgr4 exhibits 63% sequence identity to the human FCGR3A (Fc fragment of IgG receptor IIIa, CD16A)[72], which is localized at 1q23.3 on the long (q) arm of chromosome 1. As mouse FcγRIV can also bind IgE (see above), it was thought to function similarly as the human IgE receptor FcεRI [78].

Due to diverse biological functions of FcγRs, these molecules are involved in susceptibility to cancer, autoimmune, allergic and infectious diseases [79; 80], including leishmaniasis. Internalization of *Leishmania* parasites into these cells is mediated by complement receptors CR1 (CD35) and CR3 (CD11b/CD18), mannose-fucose receptor (CD206), fibronectin receptors (CD49d.CD29, CD49.e/CD29, CD41/CD61), several FcγRs (CD64, CD32, CD16) and DC-SIGN (CD209) [81]. Thus, FcγRs are directly involved in *Leishmania* infection. Their influence on susceptibility to the disease depends on genotype of the host [82; 83], on parasite species [83; 84] and likely also on the experimental design (18, 19). BALB/c mice with deleted FcγR common chain exhibited enhanced resistance to L.

major manifested by smaller skin lesions containing less parasites than wild type BALB/c [82], whereas opposite outcome was observed in analysis of FcγR knockouts on C57BL/6 genetic background [83]. On the other hand FcγR knockouts on C57BL/6 background were more resistant to *L. mexicana* [84] than wild type mice. It needs to be mentioned that the described results are not completely comparable, because in experiments leading to increased susceptibility of FcγR knockouts, parasites were intradermally injected into ear[83], whereas in experiments resulting to increased resistance of the knockouts inoculation into hind footpad was used [83; 84]. In humans, polymorphism in FCGR2A (CD32A) was associated with delayed-type hypersensitivity DTH after infection with L. infantum [85]. However, nothing is known about the role of FCGR4 in leishmaniasis.

We therefore studied expression of Fcgr4 mRNA in skin, inguinal lymph nodes, spleen and liver after *L. major* infection and in uninfected controls using two different genetic groups of mouse strains: the BALB/c-c-STS/Dem (CcS/Dem) and O20/A-c-C57BL/10-H2pz (OcB/Dem) series.

Genetic control of leishmaniasis

In early studies on leishmaniasis, the strains of mice were divided in to two main groups. In most mouse strains (e.g. A/J, C57BL/6, C57BL/10Sn, C3H, B10.D2, CcS-5 and STS/A), L. major infection causes no or only temporary pathological changes, whereas in some strains (BALB/c, SWR/J, MAI, P/J, CcS-16) the parasites disseminate from the original cutaneous site of infection and cause a systemic disease affecting skin and visceral organs [9; 15]. This indicated strong influence of the genetic background of inbred mouse strains on the outcome of leishmaniasis [86].

Despite the differences between the human disease and the mouse models, most of information obtained from the mouse is applicable to human leishmaniasis [87]. Studies of leishmaniasis in mice have been of immense interest because they allow extensive experimental manipulation and reproduce many of the features of the human disease [9; 15]. Several experimental models have been used to determine the susceptibility and resistance mechanisms triggered by infection with *L. major*. The extensive information about the genetics of leishmaniasis in mouse models revealed a multigenic basis of susceptibility, with heterogeneous effects of individual genes, and highlighted the ability to resolve genetic control at different stages of the disease [9]. This genetic evidence correlates well with the complexity of the response to infection that has been revealed by immunological studies [9]. The search for loci and genes controlling leishmaniasis included hypothesis-free genome-wide linkage analysis and association mapping, and hypothesis-based candidate gene approach.

Hypothesis-based reverse genetic or candidate gene-driven approach is based on previously identified functions and the role of a gene in the immune system. Using transgenic and knockout technologies, it includes manipulation of sequence and expression (insertion, inactivation or modification) of a specific gene in the whole animal while the rest of the genome is constant. Observation of changes of phenotype can reveal details of the gene function within pathology. Typing of candidate genes, which were chosen on the basis of previous immunological studies, 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 [9]), *TGFB1* (transforming growth factor, beta 1), *IL1*, *IL6*, *CCL2/MCP1* (chemokine (C-C motif) ligand 2), *CXCR1* (chemokine (C-X-C motif) receptor 1),

CXCR2 (chemokine (C-X-C motif) receptor 2), *FCN2* (ficolin-2) and *MBL2* (mannose-binding lectin (protein C) 2) (reviewed in [88]) on response to different human leishmaniasis.

Despite remarkable advantages of candidate gene approach, it is not suitable for the genome-wide systemic study of the complex disease regulation. Hypothesis-free forward genetic approach or phenotype-driven approach aims to identify the genetic basis of the observed phenotypes. This approach can be performed using inbred and various recombinant strains [89]. Research or phenotype variation between mouse strains allowed identifying many important principles of immunological responses, including the discovery of MHC restriction and the influence of Th1 versus Th2 responses on pathogen resistance [89; 90]. Phenotype-driven research on susceptibility genes included genome-wide linkage and association studies (GWAS). Human GWAS revealed polymorphism in *IL2RB* (interleukin 2 receptor, beta chain) [91] and *DLL1* (delta-like 1 (Drosophila)) [92] that controls susceptibility to *L. donovani* and *L. infantum chagasi* / *L. donovani* respectively. Genome-wide association study also showed that common variants in the *HLA-DRB1-HLA-DQA1* HLA class II region influenced susceptibility to *L. donovani* and *L. infantum chagasi* [93].

Genome-wide linkage scan in murine model of leishmaniasis revealed susceptibility gene *Nramp1* (Natural resistance-associated macrophage protein 1)/*Slc11a1* (solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1) [94]. The role of this gene has also been proved in human [9]. Wound healing related gene *Fli1* (friend leukaemia integration 1) was first identified to influence cutaneous leishmaniasis caused by *L. major* in mouse [95], and later also discovered to have impact on susceptibility to *L. braziliensis* caused cutaneous leishmaniasis in human [96].

Interestingly, many of the susceptibility genes or loci identified in mice were co-localized with human genes which had role in controlling severity of the disease (Table 5). Polymorphism in *IL-1β* gene was associated with susceptibility to *L. mexicana* [97] and *L. infantum* in human; it co-localized with *Lmr14* locus containing *IL-1* gene in mouse [98]. The *MIF* gene, which co-locilizes with *Lmr19* locus in mouse[99], was proven to have influence on susceptibility to *L. braziliensis* [100]. *Lmr5* on mouse chromosome 10 [101]contains *Ifng* gene, which was associated with susceptibility to *L. donovani* infection in human [102]. *CCL2* located in *Lmr15* locus in mice [98] influenced the outcome of the disease after infection with *L. braziliensis* [103]. The candidate gene *Flii* in mouse *Lmr15* was also proven to have controlling effect on human leishmaniasis after *L. braziliensis* infection [104]. Previously identified locus 5q23.3-q31 in human contains *IL-4* gene [105] which co-localized with *Lmr15* locus in mouse [98]. Mouse *Lmr6* [101] contains *Lif* gene which co-localized with the locus 22q12 in human, and might play a role in susceptibility to leishmaniasis [106; 107]. *Lmr13* susceptibility locus in murine leishmaniasis [108]contains *SMAD2, 4, 7* genes, which were proven to control susceptibility after *L. braziliensis* infection [104].

Natural polymorphisms detected in mouse genes *bg* (beige)/*Lyst* (lysosomal trafficking regulator) [109] and cationic amino acid transporter *Slc7a2* (solute carrier family 7 (cationic amino acid transporter, y+ system), member 2) [110] influenced response to *L. donovani* [111] and *L. major* [110] respectively. It remains to be tested, whether homologous genes also play role in humans.

Another strategy to study genetic control of leishmaniasis is using recombinant congenic strains (RCS) which is based on reducing the percentage of genome received from the donor strain relative to genome of the background strain, is applied in recombinant congenic strains [112; 113]. Recombinant congenic strains of the CcS/Dem series contain a different, random set of approximately 12.5% genes of the donor strain STS/A (STS) and approximately 87.5% genes of the background strain BALB/cHeA (BALB/c) [112] . The donor strain of the OcB/Dem series C57BL/10-H2pz (B10.O20) is a H2 congenic strain on the C57BL/10Sn (B10) background (N8), which carries H2pz haplotype derived from the strain O20/A (O20) [112]. Because the inbreeding of the strain B10.O20 started at the N8 generation, in addition to H2 region, approximately 1.68% of the rest of the genome is derived from the strain O20. The OcB/Dem strains OcB-9 and OcB-43 contain 12.5% or 6.25%, respectively, of non-H2 genes of the B10 strain spread in the O20 genome [112].

The genetic segment carrying controlling gene/loci can be mapped and studied separately using markers which can be typed by PCR for simple sequence length polymorphism. Totally three series of RCS were produced using different background and donor strain pairs: BALB/cHeA as a background strain and STS/A as a donor strain (CcS/Dem series) (Figure 4), C3H/DiSnA and C57BL/10ScSnA (the HcB/Dem series), and O20 and B10.O20 (the OcB/Dem series) [112; 114]. The CcS/Dem series was used in the experiments with *Leishmania* research that are included in the present thesis. An important advantage of the RC strains is random distribution of 12.5% of donor strain genes on chromosomes of background strain. These strains are genetically well characterized, homogenous and homozygous at virtually all loci. Therefore, each strain can be used to

study the genetic dissection of mechanisms of a given phenotype produced by specific genes/loci.

The application of the RCS approach to speed up mapping of QTLs responsible for human disease is of specific interest to human geneticists. As soon as a QTL is mapped in RCS an approximate chromosomal location identified loci can be found in human genome. Increasing information about homology of mouse and human genome along with well characterized phenotypes can be used to select a RCS to dissect genetic control of the phenotype and find homologous loci in human [114]. The application of RCS allowed genetic studies of various types of cancer [115; 116], cytokne-induced activation [117; 118], spontaneous proliferation of splenocytes [119], allogenic reactions [120; 121], and cytokine production [122; 123], T cell receptor induced activation [119; 124; 125], *Bordetella pertussis* infection [126], trypanosomiasis [127], tick born encephalitis [128], allergic asthma [129], and skeletal fragility [130]. In case of leishmaniasis, RC (recombinant congenic) inbred mouse strains that enabled to receive strong experimental results leading to detection of more than 20 *Lmr* (*Leishmania major* response) and 8 *Ltr* (*L. tropica* response) genetic loci especially were useful (Tabele 1)[9].

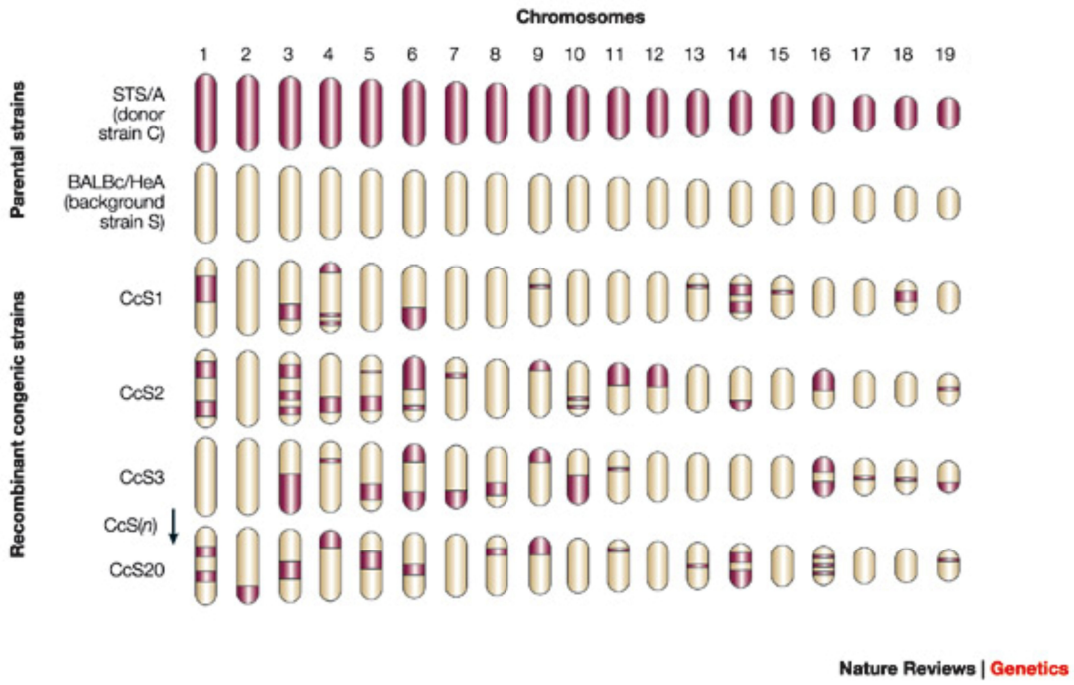

Figure 4. CcS/Dem series of recombinant congenic strains [115]

Introduction of the RCS system into leishmaniasis studies has stimulated significant progress in mapping of genes that are involved in susceptibility to *L. major*. STS/A is a resistant strain and BALB/c is a susceptible strain to leishmaniasis. Mating of a susceptible inbred strain with a resistant strain finally produced progeny that resembled either one of the parents or fall in the middle of the phenotypic spectrum which very often was different from both parents. This specific feature has made the CcS/Dem series exhibiting different range of susceptibly to leishmaniasis. CcS/Dem RCS have become a reliable experimental model of the infection which can be used to study immune pathological symptom of the disease, to map of multiple loci that determine susceptibility to various *Leishmania* species (Table 6) [101; 108; 131]. Studies of genetic control of leishmaniasis using RCS have

revealed the contribution of the individual loci to different components of the immune response and disease pathology [9; 87; 131] (Figure 5).

Table 1. Loci and candidate genes controlling leishmaniasis in mouse model

Locus or gene	Chr	*Leishmania* species	Trait controlled	candidate gene	Reference
Lsh/Nramp 1/Slc11a1	1	*L. donovani, L. infantum, L. mexicana*	parasite numbers in liver spleen	*NRAMP1*	[132; 133; 134]
Lmr14	2	*L. major*	hepatomegaly, splenomegaly, IgE, IFN-γ, IL-12, TNF-α, proliferation	*Il-1 complex*	[98; 99; 108]
Lmr14	2	*L. major*	hepatomegaly, splenomegaly, IgE, IFN-γ, IL-12, TNF-α, proliferation	*Il-1 complex*	[98; 99; 108]
Lmr16	2	*L. major*	proliferation	*CD40, Mmp9*	[99]
Lmr11	3	*L. major*	IgE, IL-6	*Il-12a, Il-6ra*	[99; 108]
Lmr3	5	*L. major*	splenomegaly, hepatomegaly, skin lesions, Ig-E, IFN-γ, proliferation	*Cd40l*	[99; 101; 108]
Lmr4	6	*L. major*	skin lesions, IFN-γ	*Il-12rb2*	[101]
Lmr17	9	*L. major*	IFN-γ, TNF-α	*Fli1, Nfrkb*	[99]
Lmr2	9	*L. major*	skin lesions	*Tirap, Fli1, Il-10r, Mmp family, Aplp2*	[95; 135]
Lmr19	10	*L. major*	proliferation	*Mif,Itgb2, Timp3*	[99]
Lmr5	10	*L. major*	skin lesions, splenomegaly, IgE, IFN-γ, IL-12	*Ifng, Stat6*	[99; 101; 108]
Lmr5	10	*L. major*	skin lesions, splenomegaly, parasite load in spleen, IgE IFN-γ, IL-4, IL-12	*Ifng*	[131]

Lmr15	11	*L. major*	hepatomegaly, IFN-γ	*Nos2, Ccl2*	[98; 99]
Lmrq5	15	*L. major*	skin lesions	*Eae2,Il7r, Lifr, C6, C7*	[136]
Lmr7	17	*L. major*	proliferation	*H2, Tnf family*	[101]
H2	17	*L. donovani*	parasite numbers in liver (strongest influence), and in spleen and bone marrow	*H2*	[133; 137; 138]
Lsh/Nramp 1/Slc11a1		*L. infantum*	parasite numbers in liver, spleen and bone marrow	*NRAMP1*	[133]
Lmr1	17	*L. major*	skin lesions	*H2, Tnf family*	[135]
Lmr13	18	*L. major*	splenomegaly	*SMADs 2, 4, 7*	[98; 99; 108]
Ltr1		*L. tropica*	parasite burden in inguinal	*Traf6, CD44, Dll1, Hdc, Il1b*	[88]
Ltr2	2	*L. tropica*	skin lesions, Parasites in liver, Hepatomegaly, splenomegaly, CCL7	*Mmp9, CD40, Ptpn1*	[88]
Ltr3	3	*L. tropica*	skin lesions, splenomegaly, CCL3, CCL5	*Il2a, Vcam1, Notch2*	[88]
Ltr4	4	*L. tropica*	skin lesions, parasite burden in liver, parasite burden in inguinal	*Ifnb, Jun*	[88]
Ltr5	10	*L. tropica*	splenomegaly	*Mif, Igf1, Stat6*	[88]
Ltr6	11	*L. tropica*	Parasites in spleen, CCL7	*Nos2, Lgals9,Ccl1, Ccl2, Ccl5, Ccl7, Ccl8, Ccl11,*	[88]
Ltr7	17	*L. tropica*	Splenomegaly, CCL3, CCL5	*Man2a1*	[88]
Ltr8	18	*L. tropica*	Splenomegaly, parasite burden in liver, CCL7	*Cd74, Mbd2, Smad7*	[88]

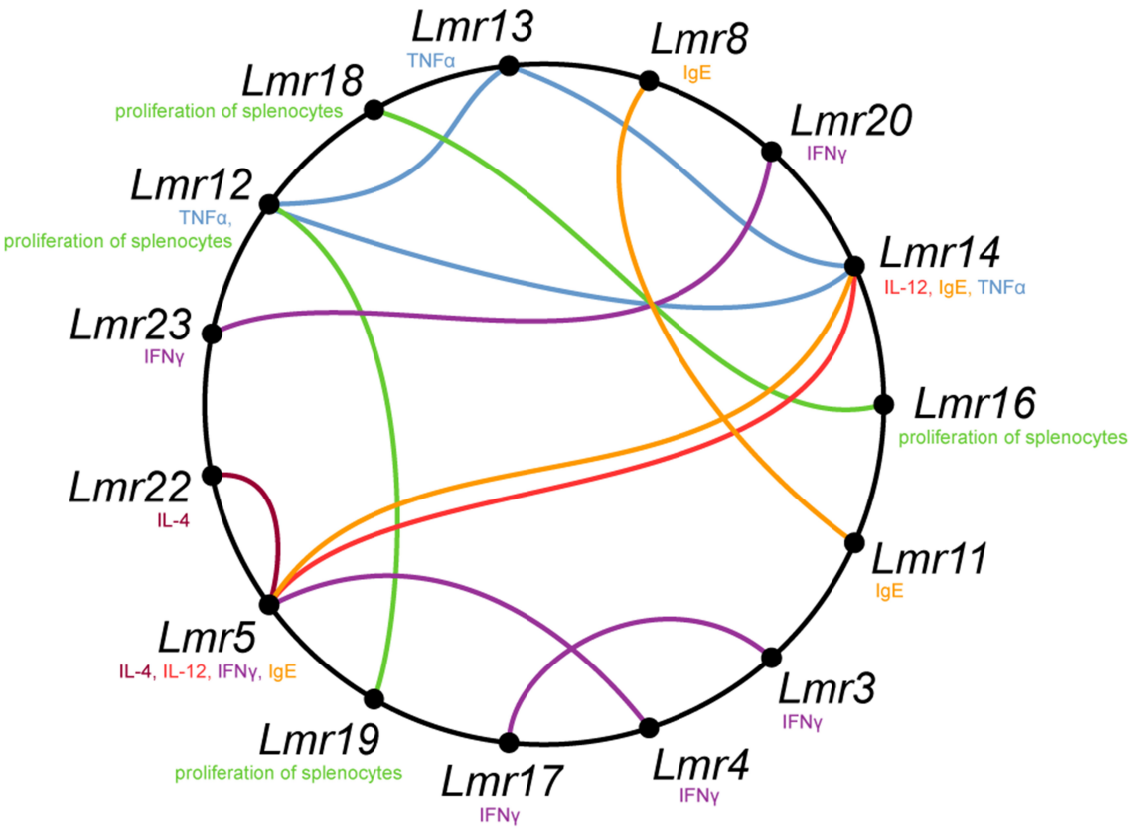

Figure 5, Complexity and gene-gene interactions in *L. major* infection

Genetic control of parasite load in organs

Parasite load is one of the most important parameters of leishmaniasis determining the course of infection and the degree of susceptibility. However, the information about genetic control of parasite load remains incomplete and fragmented; there is no systematic description of the control of parasite load in combination with other pathological parameters and influence of sex on these genes is not known for any of the studied *Leishmania* species. The use of mouse models in studies of selected candidate genes and also for hypothesis-generating genome-wide association and linkage analysis, revealed several genes and loci controlling parasite burden [9; 87; 88; 131] (**Table 2**). However, quantification of parasites had been a laborious task providing inaccurate results due to

technical problems. These problems were reduced with development of sensitive PCR-based assays [4], which permitted to perform genome wide-search [87; 88; 131].

Table 2. Genetic control of parasite load in mouse leishmaniasis

Parasite	Organ	Gene/Locus	Reference
L. donovani	Spleen	*Slc11a1* (weak effect), *H2*, *Lyst*	[111; 133]
	Liver	*Slc11a1, H2, Ir2*	[132; 133; 137; 138]
	Bone marrow	*H2*	[133]
L. mexicana	Spleen	*Slc11a1, H2*	[134]
	Liver	*Slc11a1, H2*	[134]
L. infantum	Spleen	*Slc11a1, H2*	[133]
	Liver	*Slc11a1, H2*	[133]
	Bone marrow	*H2*	[133]
L. major	Spleen	*Lmr5*	[131]
	Skin	*Dice1.2*	[139]
	Lymph nodes	*Lmr20*	[131]
L. tropica	Spleen	*Ltr3, Ltr6*	[88]
	Liver	*Ltr2, Ltr4, Ltr8*	[88]
	Lymph nodes	*Ltr1, Ltr4*	[88]

We explored genetic control of parasite load in different organs after *L. major* infection. This parasite is the predominant causative agent of human cutaneous leishmaniasis in the Old World, in rare cases it can visceralize in an immunocompromised (HIV infected) host [140], but *L. major* strain (MRHOM/IR/75/ER) was described to visceralize also in an immunocompetent individual [141]. Instances of *L. major* visceralization in non-immunocompromised people may suggest the presence of genetic factors determining extreme forms of high susceptibility to *L. major* infection. Infection by *L. major* in mouse is controlled by multiple genes. These multiple genes-loci have been mapped in three different resistant strains - C57BL/10Sn (B10.D2), C57BL/6 and STS -

using the susceptible strain BALB/c for mapping in each case [9; 87]; in cross with B10.D2 using for infection *L. major* (strain WHOM/IR/-/173), in crosses with C57BL/6 and STS *L. major* V121 (the cloned line V121 derived from MHOM/IL/67/Jericho II) and *L. major* strain (LV 561 (MHOM/IL/67/LRC-L137 JERICHO II), respectively. These experiments revealed 26 *Lmr* (*Leishmania major* response) and 5 *Lmrq* (*Leishmania major* resistance QTL) loci that determine skin lesion size, splenomegaly, hepatomegaly, cytokine levels in blood serum, eosinophil infiltration into lymphatic nodes, and parasite numbers in different organs [9; 87; 142]. Several loci (*Lmr4*/*Lmrq1*, *Lmr5*/*Lmrq3*, *Lmr6*/*Lmrq4* and *Lmr12*/*Dice1b*) detected in crosses with resistant strains STS and B10.D2 overlap, which might indicate in these loci not only general response across different mouse strains, but also general response to different *L. major* strains.

In the present study, we tested parasite load and dissemination in F_2 hybrids of BALB/c and recombinant congenic mouse strains CcS-9 and CcS-16, infected by *L. major* LV561. The disease development during previous experiments showed no significant difference between females and males of the CcS-16 strain after infection with *L. major*[143]; however the influence of sex was present in CcS-9 strain [142]. These differences determined selection of sex of mice used in the present F_2 hybrid study. The current study aims to provide a first systematic a genome wide search description of the genetic control of parasite load in mammalian organs after *L. major* infection.

Application of RCS in studying genetic control of different complex diseases has been increased during years. This approach has allowed extensive screening of segregating populations with a higher density of markers and making use of crosses with limited genetic heterogeneity. The genes/loci which are mapped using CcS/Dem RC strains which

are involved in controlling *Leishmania* infection are also shown to influence the response to other pathogens, such as *Borellia burgdorferi, Trypanosoma congolense, Salmonella typhimurium, Mycobacterium tuberculosis, Listeria monocytogenes* and *Plasmodium chabaudi* [9].

Genetic control of Tick-borne encephalitis

We also took an advantage of using RCS to study genetic control of Tick-borne encephalitis (TBE). Tick-borne encephalitis is the main tick-borne viral infection in Eurasia. It is prevalent across the entire continent from Japan to France [144]. The disease is caused by tick-borne encephalitis virus (TBEV), a flavivirus of the family Flaviviridae, which besides TBEV includes West Nile virus (WNV), dengue virus (DENV), Zika virus (ZIKV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), and several other viruses causing extensive morbidity and mortality in humans. Ticks act as both the vector and reservoir for TBEV. The main hosts are small rodents, with humans being accidental hosts. In Europe and Russia between 5000 and 13,000 clinical cases of TBE are reported annually, with a large annual fluctuation [145]. The highest incidence of TBE is reported in western Siberia, in the Czech Republic, Estonia, Slovenia and Lithuania, but the prevalence of the disease is believed to be higher than actually reported [144; 145]. TBEV may produce a variety of clinical symptoms, from an asymptomatic disease to a fever and acute or chronic progressive encephalitis. The outcome of infection depends on the strain of virus [144], as well as on the genotype [146], sex and age of the host [147], and on the environmental and social factors [144]. Environmental and social factors influence also risk of infection.

Genetic influence on susceptibility to TBEV-induced disease has been analyzed by two main strategies: a hypothesis-independent phenotype-driven approach and a hypothesis-driven approach. Application of a genome-wide search (hypothesis-independent approach) in mouse led to identification of the gene Oas1 (2′-5′-oligoadenylate synthetase gene) [148; 149]. A stop codon in exon 4 of the gene Oas1b (a natural knockout) present in majority of mouse laboratory strains causes production of protein lacking 30% of the C terminal sequence [148]. This part of molecule seems to be critical for tetramerization required for OAS1B activity leading to degradation of viral RNA. Thus, this mutation makes majority of mouse laboratory strains susceptible to flaviviruses [149; 150]. Human ortholog to this gene (OAS1) also modifies susceptibility to other flaviviruses (WNV) [151; 152], whereas OAS2 and OAS3 localized in the same cluster on chromosome 12q24.2 influence response to TBEV [146]. The polymorphic sites associated in OAS2 and OAS3 with susceptibility to TBEV did not resulted in amino acid changes, thus mechanisms of susceptibility control is not known [146]. The hypothesis-driven approach has focused on genes that encode molecules indicated to be involved in antiviral response by mechanistic studies [152]. These candidate genes studies revealed that polymorphisms in CD209/DC-SIGN [153], CCR5 [154], TLR3 [155], IL10 [156] and IFNL3/IL28B [156] influence susceptibility to TBEV in humans.

Our previous study has shown that both after subcutaneous and intracerebral inoculation of European prototypic TBEV, BALB/c mice exhibited intermediate susceptibility to the infection, STS mice were highly resistant, whereas the strain CcS-11, which carries 12.5% of the STS genome on the background of the genome of the strain BALB/c [112], is even more susceptible than its two parents—BALB/c and STS [128].

Importantly, mouse orthologs of human TBEV controlling genes: Oas1b, Cd209, Tlr3, Ccr5, Il10 and Ifnl3 are in CcS-11 localized on segments derived from the strain BALB/c , so they are identical in both BALB/c and CcS-11 and hence cannot be responsible for the phenotypic difference of the two strains. Therefore, the difference must be due to a presently unknown locus, which could be detected by a linkage study of a cross between BALB/c and CcS-11. Thus, we have generated a F2 intercross between BALB/c and CcS-11 and performed a linkage and bioinformatics analysis.

Aims of the study

This thesis has three major aims:

i) To find novel mechanisms influencing *Leishmania* infection and to establish whether they are regulated by the genetic background of the mammalian host

ii) Mapping new genes controlling parasite load after *L. major* infection

iii) To establish genetic control of survival after infection with tick-borne encephalitis virus and to determine its relationship to genetic control of leishmaniasis

Organization of the thesis:

i) Novel mechanisms influencing *Leishmania major* infection

Experimental studies:

Sohrabi Y, Volkova V, Kobets T, Havelková H, Krayem I, Slapničková M, Demant P, Lipoldová M. Genetic Regulation of Guanylate-Binding Proteins 2b and 5 during Leishmaniasis in Mice. Front Immunol. 2018;9:130.

Havelková H, Sohrabi S, Volkova V, Krayem I, Slapničková M, Demant D, LipoldováM, Genetic regulation of Fcγ receptor IV in leishmaniasis, submitted to Front Immunol (Under revision)

Theoretical analysis:

Sohrabi Y*, Lipoldová M. Mannose Receptor and the Mystery of Nonhealing *Leishmania major* Infection. Trends Parasitol. 2018 May;34(5):354-356.

Dorhoi A, Glaría E, Garcia-Tellez T, Nieuwenhuizen NE, Zelinskyy G, Favier B, Singh A, Ehrchen J, Gujer C, Münz C, Saraiva M, Sohrabi Y, Sousa AE, Delputte P, Müller-Trutwin M, Valledor AF. MDSCs in infectious diseases: regulation, roles, and readjustment. Cancer Immunol Immunother. 2018 Dec 19. doi: 10.1007/s00262-018-2277-y.

ii) Mapping new genes controlling parasite load after *L. major* infection

Kobets T, Cepickova M, Volkova V, Sohrabi Y, Havelková H, Svobodova M, Demant P and Lipoldova M. Genetic Architecture of Control of Parasite Load in Organs of Mice Infected with Leishmania major: Gene-Gene Interactions and Sex Influence. Front Immunol. 2019

iii) To establish genetic control of survival after infection with tick-borne encephalitis virus and to determine its relationship to genetic control of leishmaniasis

Palus M*, Sohrabi Y*, Broman KW, Strnad H, Šíma M, Růžek D, Volkova V, Slapničková M, Vojtíšková J, Mrázková L, Salát J, Lipoldová M. A novel locus on mouse chromosome 7 that influences survival after infection with tick-borne encephalitis virus. BMC Neurosci. 2018 Jul 6;19(1):39.

Chapter 2; summary of the results and discussion

I) Novel mechanisms influencing *Leishmania major* infection

-Experimental studies:

i) Genetic regulation of guanylate binding proteins 2b and 5 during leishmaniasis in mice (Supplement 1)

Interferon-induced GTPases (guanylate-binding proteins, GBPs) play an important role in inflammasome activation and mediate innate resistance to many intracellular pathogens, but little is known about their role in leishmaniasis. We therefore studied expression of *Gbp2b*/*Gbp1* and *Gbp5* mRNA in skin, inguinal lymph nodes, spleen and liver after *Leishmania major* infection and in uninfected controls. For our analysis we selected two murine Gbps with the C-terminal CaaX sequence enabling targeting proteins to parasitophorous membranes [44]. We studied expression of *Gbp2b/Gbp1* and *Gbp5* in vivo before and 8 weeks after *L. major* infection in ten mouse strains from two genetically distant but internally related groups: CcS/Dem (BALB/c, STS, CcS-5, CcS-16, CcS-20) and OcB/Dem (O20, C57BL/10 (B10), C57BL/10-H2pz (B10.O20), OcB-9, OcB-43).

Genetic influence on expression of *Gbp2b/Gbp1* and *Gbp5*

Tested strains exhibited genetic differences in *Gbps* expression both before and after *L. major* infection. Our study extends analysis of genetic influence by Staeheli and coworkers on Gbp2b/Gbp1 expression[157], who injected forty six mouse strains by poly(I);poly(C) in order to induce interferon production and tested their spleen cells for guanylate binding activity. Tested strains were divided into Gbp2b/Gbp1 inducible and

Gbp2b/Gbp1 non-inducible groups. BALB/c was in the inducible group, whereas STS, O20, and C57BL/6J belonged to non-inducible one [157]. Our data confirm strong genetic influence on expression of *Gbp2b/Gbp1*; however a direct comparison of outcome of study of Staeheli and coworkers [157] with our results is impossible due to different experimental designs. They induced Gbp2b/Gbp1 expression by poly(I);poly(C) that is structurally similar to double-stranded RNA present in some viruses, whereas we stimulated *Gbp2b*/*Gbp1* expression by the chronic infection with parasite *L. major*.

Comparison of genotypes in Gbp cluster on mouse chromosome 3 indicates trans-regulation

The limited and defined genetic differences between strains in each group (33) make it possible to identify the differences in *Gbp* expression that are controlled by genes outside the *Gbp* coding gene-complex on chromosome 3. Incidence and size of skin lesions indicate that BALB/c and CcS-16 are highly susceptible and B10.O20 is susceptible to *L. major*; whereas CcS-20 is intermediate and STS, CcS-5, O20, B10, OcB-9, OcB-43 are resistant to this parasite (34, this study). The strains were classified on basis of size and number of infection-induced skin lesions as highly susceptible (BALB/c, CcS-16), susceptible (B10.O20), intermediate (CcS-20), and resistant (STS, O20, B10, OcB-9, OcB-43). Comparison of genotypes of the tested strains revealed that strains CcS-5, CcS-16 and CcS-20 that exhibited higher expression of Gbp had *Gbp* genotype identical to that of BALB/c (C). Similarly, highly differing CcS-5 and CcS-16 strains carry the same *Gbp* allele. The presence of the same allele of *Gbp* gene cluster on chromosome 3 in strains that differ in other genes suggests that their differences in expression of *Gbp2*/*Gbp1* and/or *Gbp5* from one or both parents or from other RC strain are due to regulatory influence of non-

Gbp gene(s) of STS origin carried on other genetic segments (*trans*-regulation). In the OcB/Dem series, B10.O20 carried in *Gbp* cluster B10 genotype (B), which similarly indicated trans-regulation of expression from O20 genome situated outside *Gbp* cluster. This *trans*-regulation can be partly overlaid by other regulatory events appearing after infection. Further genetic studies will be needed to elucidate nature of regulatory events observed in our studies.

Increased expression of *Gbp2b/Gbp1* and/or *Gbp5* in resistant mice suggests hidden inflammation

Infection resulted in approximately 10x upregulation of *Gbp2b*/*Gbp1* and *Gbp5* mRNAs in organs of both susceptible and resistant strains, which was most pronounced in skin. CcS-20 expressed higher level of *Gbp2b*/*Gbp1* than both parental strains in skin, whereas CcS-16 expressed higher level of *Gbp2b*/*Gbp1* than both parental strains in skin in liver. This indicates a trans-regulation present in infected mice CcS-16 and CcS-20.

Immunostaining of skin of five strains revealed in resistant and intermediate strains STS, CcS-5, O20, and CcS-20 tight co-localization of Gbp2b/Gbp1 protein with most *L. major* parasites, whereas in the highly susceptible strain BALB/c most parasites did not associate with Gbp2b/Gbp1. This strongly suggests that persistent parasites can contribute to the maintenance of protective immunity, which was manifested in our experiments by the increased levels of Gbp2b/Gbp1 and Gbp5 in resistant mice.

ii) Genetic regulation of Fcγ receptor IV in leishmaniasis (Supplement 2)

Fcγ receptor IV (FCGR4), the receptor for the Fc fragment of immunoglobulin G (IgG), participates in IgG2a- and IgG2b-dependent effector functions in autoimmune responses and antibody-dependent cellular cytotoxicity in mouse. Experiments with mice bearing knockouts of other Fcγ receptors have shown that the genetic background of the host controls their role in response to *Leishmania* parasites, leading either to development of protective immunity or to aggravation of disease manifestations. There is also genetic evidence that human gene *FCGR2A* might play a role in IgG-mediated inflammation in asymptomatic *L. infantum* infection. However, the information about the role of FCGR4 in leishmaniasis is missing. We therefore studied expression of *Fcgr4* mRNA in skin, inguinal lymph nodes, spleen and liver after *L. major* infection and in uninfected controls. We tested two genetic series of mouse strains: BALB/c, STS and CcS-5, CcS-16 and CcS-20 that consist of different combinations of BALB/c and STS genomes, and strains O20, C57BL/10 (B10) and B10.O20, OcB-9 and OcB-43 carrying different mixtures of O20 and B10 genomes. BALB/c and CcS-16 are highly susceptible, B10.O20 is susceptible, CcS-20 shows intermediate pathology and strains STS, CcS-5, O20, B10, OcB-9, OcB-43 are resistant and do not exhibit signs of clinical manifestations of the disease despite the presence of parasites in their organs.

Strong genetic influence on expression of *Fcgr4* mRNA in uninfected mice

Analysis of expression of *Fcgr4* in skin, inguinal lymph nodes, spleen and liver of uninfected mice within two genetically different series of mouse strains: CcS/Dem (BALB/c, STS, CcS-5, CcS-16, CcS-20) and OcB/Dem (O20, B10, B10.O20, OcB-9, OcB-43) revealed strong genetic influence on mRNA level in every tested organ in at least one of the

tested series. Comparison of genotypes in mouse *Fcgr* cluster on the distal mouse chromosome 1 revealed that CcS-20 carries the same *Fcgr4* allele as BALB/c, and OcB-9 the same *Fcgr4* allele as O20. Such differences are believed to be due to trans-regulation by distant genes. Thus, the presence of this *Fcgr4* allele of the chromosome 1 in strains differing at other parts of the genome suggests that their *Fcgr4* expression from their parental strains reflect regulation by STS derived non-*Fcgr4* origin located in other genomic regions (*trans*-regulation). Similarly, OcB-9 has *Fcgr* allele of O20 (O), which indicated a likely trans-regulation of expression by B10-derived genes outside *Fcgr* cluster. In uninfected mice we observed in total 11 instances of *trans*-regulation**.** Uninfected BALB/c and STS did not differ in their expression in skin, but in CcS-20, carrying BALB/c-derived *Fcgr4* gene, expression of *Fcgr4* exceeds that of both parents. These data suggests *trans*-regulation of *Fcgr4*.

Infection caused a varying degree of up-regulation (up to 50x) of *Fcgr4* in organs of mouse strains irrespective of their susceptibility or resistance. It was most pronounced in skin, where all tested strains except OcB-9 exhibited increase of *Fcgr4* mRNA. *Trans*-regulation was observed also in infected mice.

Immunohistochemistry analysis confirmed up-regulation of *FCGR4* is livers of L. major infected mice

As the next step we wanted to establish whether the *Fcgr4* mRNA is translated into protein. We were also intrigued what the relationship between FCGR4 and *L. major* parasites is. To find the answer, we have compared localization of the parasites and FCGR4, as well as activated macrophages and Ly6G+ granulocytes in livers of uninfected and infected BALB/c and STS. Uninfected livers of both BALB/c (A) and STS (B) contained

relatively low levels of FCGR4 and activated macrophages and a few Ly6G+ cells. FCGR4 was present not only on activated macrophages, but also on other cell sub-populations; relatively few FCGR4 molecules were present on Ly6G+ cells. BALB/c mice contain higher numbers of *L. major* in liver than STS mice. This infection induced higher levels of FCGR4, which partly co-localized with activated macrophages and *Leishmania* parasites.

We present the first data on expression of *Fcgr4* during leishmaniasis. They revealed a strong genetic control of *Fcgr4* expression in uninfected as well as *L. major* infected mice. In certain strains it involved also trans-regulation by genes that are not closely linked to *Fcgr4*. In several organs, expression of *Fcgr4* in recombinant congenic strains were outside the range of the parental strains. Interestingly, the *Fcgr4* cluster is linked to the *Lmr20* locus on chromosome 1 that significantly influences the susceptibility to *L. major* infection [131]. This data may help to identify and manipulate these as yet unknown genes.

-Theoretical analysis:

i) Mannose Receptor and the Mystery of Nonhealing *Leishmania major* Infection (Supplement 3)

Scientists have long puzzled over the ability of *Leishmania major* Seidman (LmSd) to form nonhealing cutaneous lesions in the face of a strong Th1 response. A recent study identified a population of dermal macrophages that are preferentially infected by LmSd in a mannose receptor 1-, C-type 1(MRC1/CD206)-dependent manner. The study by Lee *et al*. [158]provides new insights into the longstanding puzzle of nonhealing infection with the *L. major* strain LmSd. As yet unidentified adaptations by this parasite promote a sustained, long-term persistence in the dermis that is resistant to the strong Th1 response. This suggests that the LmSd parasite may have evolved by changing its cell-surface oligosaccharide structures to infect and survive in a specific niche – the MRC1hi P4 dermal macrophages. Looking forward, it will be interesting to identify the LmSd epitope responsible for the invasion of MRC1hi cells and to establish whether these or different mechanisms are responsible for other nonhealing leishmaniases.

ii) MDSCs in infectious diseases: regulation, roles, and readjustment (Supplement 4).

Many pathogens, ranging from viruses to multicellular parasites, promote expansion of MDSCs, which are myeloid cells that exhibit immunosuppressive features. The roles of MDSCs in infection depend on the class and virulence mechanisms of the pathogen, the stage of the disease, and the pathology associated with the infection. This work compiles evidence supported by functional assays on the roles of different subsets of MDSCs in acute and chronic infections, including pathogen-associated malignancies, and discusses strategies to modulate MDSC dynamics to benefit the host.

Novel loci controlling parasite load in organs of mice infected with *Leishmania major*, their interactions and sex influence (Supplement 5)

The development of its most aggressive form, visceral leishmaniasis, which is lethal if untreated, is not yet understood. Visceral leishmaniasis is caused by infection and inflammation of internal organs. Therefore we analyzed the genetics of parasite load, spread to internal organs, and ensuing visceral pathology. Using a new PCR-based method of quantification of parasites in tissues we describe a network-like set of interacting genetic loci that control parasite load in different organs. Quantification of *Leishmania* parasites in lymph nodes, spleen and liver of from infected F_2 hybrids between BALB/c and recombinant congenic strains CcS-9 and CcS-16 allowed us to map two novel parasite load controlling *Leishmania major response* loci, *Lmr24* and *Lmr27*.

Control of Parasite Load in Organs: Multiple Genes and Distinct Organ Specificities That Operate in a Network with Many Gene-Gene Interactions

We also detected parasite-controlling role of the previously described loci *Lmr4*, *Lmr11*, *Lmr13*, *Lmr14*, *Lmr15* and *Lmr25,* and describe 8 genetic interactions between them. *Lmr14*, *Lmr15*, *Lmr25* and *Lmr27* controlled parasite load in liver and lymph nodes. In addition, *Leishmania* burden in lymph nodes but not liver was influenced by *Lmr4* and *Lmr24*. In spleen, parasite load was controlled by *Lmr11* and *Lmr13*.

Relationship between control of parasite load, organ pathology, and systemic immune response

We assessed relationship between parasite control, organ pathology, and systemic immune response and found a large heterogeneity in effects of controlling loci. Moreover, control of parasite load was linked with control of organ pathology in some loci, but not in others. Some loci, such as *Lmr13* and *Lmr14* carried by CcS-16, and *Lmr15* and *Lmr24* carried by CcS-9 controlled both parasite loads in organs, organ pathology and systemic

immune response: *Lmr13* determined parasite load in spleen and liver, skin lesions [98], IgE in serum [108], TNFα in serum [99]; *Lmr14* (CcS-16) influenced parasite load in liver (suggestive linkage), splenomegaly, hepatomegaly [98], IgE [108], IFNγ, IL-12, and TNFα in serum, and spontaneous proliferation of splenocytes from infected mice [99]; *Lmr15* (CcS-9) controlled parasite load in lymph nodes and in liver, infiltration of eosinophils into lymph nodes [143], skin lesions, hepatomegaly, IL-4 and IgE in serum; and *Lmr24* controlled parasite load in lymph nodes, skin lesions, splenomegaly and IL-4, IL-10, and IFNγ in serum). *Lmr14* carried by CcS-9 determined parasite load in lymph nodes and in liver and systemic immune response (IL-13 in serum), but no organ pathology. *Lmr15* carried by CcS-16 did not influence parasite load, but controls hepatomegaly [98] and IFNγ in serum [99].

Sex-Dependent Control of Parasite Load

Control of parasite load in inguinal lymph nodes is in many cases sex dependent. In the strain CcS-9, *Lmr4*, and *Lmr27* controlled parasite load in males only; *Lmr15* controlled parasite load both in females and males, but with the opposite direction: BALB/c (C) allele is linked with higher parasite load in females and lower parasite load in males. Sex has been found to influence susceptibility to many diseases, including leishmaniasis [159; 160] We also mapped additional genes controlling splenomegaly and hepatomegaly. This resulted in a systematized insight into genetic control of spread and load of *Leishmania* parasites and visceral pathology in the mammalian organism.

A novel locus on mouse chromosome 7 that influences survival after infection with tick‑borne encephalitis virus (Supplement 6)

Genetic influence on susceptibility to TBEV-induced disease has been analyzed by two main strategies: a hypothesis-independent phenotype-driven approach and a hypothesis-driven approach. Our previous study has shown that both after subcutaneous and intracerebral inoculation of European prototypic TBEV, BALB/c mice exhibited intermediate susceptibility to the infection, STS mice were highly resistant, whereas the strain CcS-11, which carries 12.5% of the STS genome on the background of the genome of the strain BALB/c, is even more susceptible than its two parents—BALB/c and STS [9]. Importantly, mouse orthologs of human TBEV controlling genes: Oas1b, Cd209, Tlr3, Ccr5, Il10 and Ifnl3 are in CcS-11 localized on segments derived from the strain BALB/c , so they are identical in both BALB/c and CcS-11 and hence cannot be responsible for the phenotypic difference of the two strains. Therefore, the difference must be due to a presently unknown locus, which could be detected by a linkage study of a cross between BALB/c and CcS-11. Thus, we have generated a F2 intercross between BALB/c and CcS-11 and performed a linkage and bioinformatics analysis. These studies revealed a novel suggestive locus on mouse chromosome 7 containing 9 potential candidate genes. Binary trait linkage analysis revealed a suggestive locus on chromosome 7 near D7Nds5 affecting the binary trait (death/survival) (LOD = 2.15), with a corresponding genome-scan-adjusted P value = 0.12. To determine the location of the STS genes responsible for susceptibility of CcS-11, we analyzed survival of TBEV-infected F2 hybrids between BALB/c and CcS-11. CcS-11 carries STS-derived segments on eight chromosomes. These were genotyped in the F2 hybrid mice and their linkage with survival was tested by binary trait interval mapping. We have sequenced genomes of BALB/c and STS using next generation sequencing and

performed bioinformatics analysis of the chromosomal segment exhibiting linkage with TBEV survival.

Linkage analysis revealed a novel suggestive survival-controlling locus on chromosome 7 linked to marker D7Nds5 (44.2 Mb). Analysis of this locus for polymorphisms between BALB/c and STS that change RNA stability and genes' functions led to detection of 9 potential candidate genes: Cd33, Klk1b22, Siglece, Klk1b16, Fut2, Grwd1, Abcc6, Otog, and Mkrn3. One of them, Cd33, carried a nonsense mutation in the STS strain. The robust genetic system of recombinant congenic strains of mice enabled detection of a novel suggestive locus on chromosome 7.

List of publications

1- Sohrabi Y, Volkova V, Kobets T, Havelková H, Krayem I, Slapničková M, Demant P, Lipoldová M. Genetic Regulation of Guanylate-Binding Proteins 2b and 5 during Leishmaniasis in Mice. Front Immunol. 2018 Feb 7;9:130.

IF: 5.511

2-Havelková H, Sohrabi S, Volkova V, Krayem I, Slapničková M, Demant D, LipoldováM, Genetic regulation of Fcγ receptor IV in leishmaniasis, submitted to Front Immunol (Under revision), **I designed, planned and performed parasitology and expression experiments and contributed to the writing of the manuscript**

IF: 5.511

3-Sohrabi Y*, Lipoldová M*. Mannose Receptor and the Mystery of Nonhealing *Leishmania major* Infection. Trends Parasitol. 2018 May;34(5):354-356.

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IF: 7.929

4-Dorhoi A, Glaría E, Garcia-Tellez T, Nieuwenhuizen NE, Zelinskyy G, Favier B, Singh A, Ehrchen J, Gujer C, Münz C, Saraiva M, Sohrabi Y, Sousa AE, Delputte P, Müller-Trutwin M, Valledor AF. MDSCs in infectious diseases: regulation, roles, and readjustment. Cancer Immunol Immunother. 2018 Dec 19. doi: 10.1007/s00262-018-2277-y. **I contributed to the writing of the manuscript**

IF: 4.846

5-Kobets T, Cepickova M, Volkova V, Sohrabi Y, Havelková H, Svobodova M, Demant P and Lipoldova M. Genetic Architecture of Control of Parasite Load in Organs of Mice Infected with *Leishmania major*: Gene-Gene Interactions and Sex Influence. Front Immunol. 2019, **I contributed in performing experiments**

IF: 5.511

6- Palus M*, Sohrabi Y*, Broman KW, Strnad H, Šíma M, Růžek D, Volkova V, Slapničková M, Vojtíšková J, Mrázková L, Salát J, Lipoldová M. A novel locus on mouse chromosome 7 that influences survival after infection with tick-borne encephalitis virus. BMC Neurosci. 2018 Jul 6;19(1):39.

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Genetic Regulation of Guanylate-Binding Proteins 2b and 5 during Leishmaniasis in Mice

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Interferon-induced GTPases [guanylate-binding proteins (GBPs)] play an important role in inflammasome activation and mediate innate resistance to many intracellular pathogens, but little is known about their role in leishmaniasis. We therefore studied expression of *Gbp2b/Gbp1* and *Gbp5* mRNA in skin, inguinal lymph nodes, spleen, and liver after *Leishmania major* infection and in uninfected controls. We used two different groups of related mouse strains: BALB/c, STS, and CcS-5, CcS-16, and CcS-20 that carry different combinations of BALB/c and STS genomes, and strains O20, C57BL/10 (B10) and B10.O20, OcB-9, and OcB-43 carrying different combinations of O20 and B10 genomes. The strains were classified on the basis of size and number of infection-induced skin lesions as highly susceptible (BALB/c, CcS-16), susceptible (B10.O20), intermediate (CcS-20), and resistant (STS, O20, B10, OcB-9, OcB-43). Some uninfected strains differed in expression of *Gbp2b/Gbp1* and *Gbp5*, especially of *Gbp2b/Gbp1* in skin. Uninfected BALB/c and STS did not differ in their expression, but in CcS-5, CcS-16, and CcS-20, which all carry BALB/c-derived *Gbp* gene-cluster, expression of *Gbp2b/Gbp1* exceeds that of both parents. These data indicate *trans*-regulation of *Gbp*s. Infection resulted in approximately 10× upregulation of *Gbp2b/Gbp1* and *Gbp5* mRNAs in organs of both susceptible and resistant strains, which was most pronounced in skin. CcS-20 expressed higher level of *Gbp2b/Gbp1* than both parental strains in skin, whereas CcS-16 expressed higher level of *Gbp2b/Gbp1* than both parental strains in skin and liver. This indicates a *trans*-regulation present in infected mice CcS-16 and CcS-20. Immunostaining of skin of five strains revealed in resistant and intermediate strains STS, CcS-5, O20, and CcS-20 tight co-localization of Gbp2b/Gbp1 protein with most *L. major* parasites, whereas in the highly susceptible strain, BALB/c most parasites did not associate with Gbp2b/Gbp1. In conclusion, expression of *Gbp2b/Gbp1* and *Gbp5* was increased even in organs of clinically asymptomatic resistant mice. It suggests a hidden inflammation, which might contribute to control of persisting parasites. This is supported by the co-localization of Gbpb2/Gbp1 protein and *L. major* parasites in skin of resistant and intermediate but not highly susceptible mice.

Keywords: *Leishmania major*, recombinant congenic strains, guanylate-binding proteins, a hidden inflammation, genetic control

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INTRODUCTION

Guanylate-binding proteins (GBPs) are components of cell-autonomous immunity playing a key role in response to intracellular infections [reviewed in Ref. (1–3)]. Besides their role in defense against pathogens, they influence cellular proliferation, adhesion, and migration [reviewed in Ref. (4)], and some members have direct anti-tumorigenic effect on tumor cells (5). GBPs and Gbps were first detected as a 67 kDa protein fraction after stimulation of different human and mouse cell lines with IFN (6) and further characterized as a GBP after stimulation of human and mouse fibroblasts with IFNα, IFNβ, and IFNγ (7). There are currently seven GBPs known in humans (encoded by genes located on the chromosome 1) [reviewed in Ref. (3, 8)] and 11 Gbps in mouse. *Gbp2b*/*Gbp1*, *Gbp2*, *Gbp3*, *Gbp5*, and *Gbp7* map to chromosome 3, whereas *Gbp4*, *Gbp6*, *Gbp8*, *Gbp9*, *Gbp10*, and *Gbp11* are localized on chromosome 5 (9). These proteins are highly conserved and belong to dynamin superfamily—multidomain mechano-chemical GTPases, which are implicated in nucleotide-dependent membrane remodeling events (10, 11).

Guanylate-binding proteins consist of an N-terminal α, β globular large GTPase domain and a α-helical finger-like C-terminal regulatory domain. The domains are connected by a short intermediate region consisting of one α-helix and a short two-stranded β-sheet (12, 13). A GTPase-domain binds guanine nucleotides with low affinities. This induces nucleotide dependent GBP multimerization and hydrolysis of GTP via GDP to GMP [reviewed in Ref. (3)]. Human GBP1, GBP2, and GBP5 and murine Gbp2b/Gbp1, Gbp2, and Gbp5 have at the C-terminus a CaaX sequence (C—cysteine, aa two amino acids, X—terminal amino acid), which directs isoprenylation—the addition of lipid moiety to the protein, which targets proteins to intracellular membranes and facilitates protein-protein interaction (4). Recruitment of proteins to parasitophorous vacuoles harboring pathogens can lead to restriction of pathogen proliferation (14).

GBPs are involved in regulation of inflammasomes—a high-molecular-weight complexes present in the cytosol of stimulated immune cells that mediate the activation of inflammatory caspases resulting in pathogen clearance and/or death of infected cell [reviewed in Ref. (1, 3, 15)]. Gbps can also attack parasites directly via supramolecular complexes (16) and interfere with virus replication (17) or virion assembly (18). Type of effective defense depends on pathogen involved.

A wide range of studies revealed an important role of GBPs in response to different infections including viral (17–20), bacterial (21–24), and protozoan pathogens (14, 16, 25), both vacuolar (14, 16, 21, 24, 25) and cytosolic (17–20).

Abbreviations: Gbp, guanylate-binding protein (murine); GBP, guanylate-binding protein (human); *Gbp2b*/*Gbp1*, murine gene coding this guanylate-binding protein was originally named *Gbp1* and later renamed *Gbp2b*; RCS, recombinant congenic strains; CcS—BALB/c-c-STS, series of recombinant congenic containing random 12.5% of genome of the donor strain STS/A (STS) on 87.5% genome of the background strain BALB/cHeA (BALB/c); OcB—O20-c-C57BL/10-*H-2pz* (B10.O20/Dem), series of recombinant congenic containing random 12.5% (or 6.25% or less) of genome of the donor strain B10.O20/Dem (B10.O20) on the background strain O20/A (O20).

For example, in human GBP1 influences resistance to vesicular stomatitis virus (19), encephalomyelocarditis virus (19), influenza A viruses (17), and *Chlamydia trachomatis* (22), GBP3 reduces virus titers of influenza A viruses (17) and GBP5 prevents processing and incorporation of the viral glycoprotein Env of HIV-1 (18).

Murine Gbp2b/Gbp1 plays role in defense against *Listeria monocytogenes* and *Mycobacterium bovis* BCG (23), Gbp2 inhibits replication of vesicular stomatitis virus and encephalomyelocarditis virus (20), *Toxoplasma gondii* (14), and *Salmonella typhimurium* (24), and Gbp5 protects against *S. typhimurium* (21) and *M. bovis* BCG (23). Moreover, several Gbps can cooperate for more effective defense. Gene specific-silencing using siRNA established that murine Gbp2b/Gbp1, Gbp5, Gbp7, and Gbp6/10 protect against *M. bovis* BCG and *L. monocytogenes*. A combination of siRNAs exacerbated the loss of function, which indicated that protective Gbps functioned cooperatively (23). Similarly, mutual molecular interactions of murine Gbp2b/Gbp1, Gbp2, Gbp3, Gbp5, and Gbp6 protected against *T. gondii* (16).

Leishmania is an obligatory intracellular mammalian pathogen that enters skin by the bite of female phlebotomine sand flies and infects so-called professional phagocytes (neutrophils, monocytes, and macrophages), as well as dendritic cells and fibroblasts. The major host cell is the macrophage where parasites reside inside parasitophorous vacuole, multiply, eventually rupturing the cell and spread to uninfected cells. Infected cells can spread to lymph nodes, spleen, liver, bone marrow, and sometimes lungs [reviewed in Ref. (26)]. The infection can remain asymptomatic or result in one of three main clinical syndromes: the cutaneous form of the disease in dermis, which can be localized or diffuse; mucocutaneous leishmaniasis in the mucosa and the visceral leishmaniasis characterized by splenomegaly and hepatomegaly that results from the metastatic spread of infection to the spleen and liver (27, 28). Manifestations of the disease depend on the infecting species, environmental and social factors, and the genotype of the mammalian host [reviewed in Ref. (26)].

There is very little known about a possible role of GBPs in *Leishmania* infection. Analysis of global gene expression of bone marrow derived macrophages from BALB/c mouse demonstrated upregulation of expression of *Gbp2b*/*Gbp1*, *Gbp2*, *Gbp3*, *Gbp6*, and *Gbp7* after 24 hours of infection with *Leishmania major* promastigotes (29). Dendritic cells generated from blood of healthy human donors exhibited increased expression of *GBP1* and *GBP2* 16 hours after infection by *L. major* promastigotes, whereas dendritic cells infected by *Leishmania donovani* had increased expression of *GBP1* (30). Comparison of global gene expression in skin lesions of *Leishmania braziliensis*-infected patients with skin of normal skin biopsies revealed upregulation of *GBP5* mRNA (31).

For our analysis, we selected two murine *Gbps* with the C-terminal CaaX sequence enabling targeting proteins to parasitophorous membranes (4). We studied expression of *Gbp2b*/*Gbp1* and *Gbp5 in vivo* before and 8 weeks after *L. major* infection in 10 mouse strains from two genetically distant but internally related groups: CcS/Dem (BALB/c, STS, CcS-5, CcS-16, CcS-20) and OcB/Dem (O20, C57BL/10 (B10), C57BL/10-*H2*^{pz}

(B10.O20), OcB-9, OcB-43). 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 (32, 33). OcB/Dem strains were derived from strains B10.O20 and O20. Strains OcB-43 and OcB-9 contain different 4 and 12.5% of B10 genome on O20 background, respectively; strain B10.O20 contains 4% of O20 genome on B10 background (32, 33). The limited and defined genetic differences between strains in each group (33) make it possible to identify the differences in *Gbp* expression that are controlled by genes outside the *Gbp* coding gene-complex on chromosome 3. Incidence and size of skin lesions indicate that BALB/c and CcS-16 are highly susceptible and B10.O20 is susceptible to *L. major*; whereas CcS-20 is intermediate and STS, CcS-5, O20, B10, OcB-9, and OcB-43 are resistant to this parasite (34) (this study).

We found that the levels of *Gbp2b/Gbp1* and *Gbp5* mRNAs are influenced by *L. major* infection and by genome of the host. The infection caused a large increase of *Gbp2b/Gbp1* and *Gbp5* expression, but *Gbp*s levels in both uninfected and infected mice differ among mouse strains indicating influence of genetic factors. These genetic influences are different in uninfected and infected mice and in some strains there is a clear evidence for a regulation by genes other than the *Gbp* genes (*trans*-regulation). We also show that Gbp2b/Gbp1 protein and *L. major* parasites co-localize in resistant strains STS, CcS-5, and O20 and in the intermediate strain CcS-20 but not in the highly susceptible strain BALB/c.

MATERIALS AND METHODS

Mice

mRNA Expression Experiments

A total of 275 (152 infected and 123 uninfected) female mice of strains BALB/c (33 infected and 22 uninfected), STS (20 infected and 13 uninfected), CcS-5 (11 infected and 10 uninfected), CcS-16 (10 infected and 11 uninfected), CcS-20 (12 infected and 12 uninfected), O20/A (O20) (12 infected and 12 uninfected), C57BL/10Sn (B10) (17 infected and 10 uninfected), B10.O20/R164/Dem (B10.O20) (17 infected and 12 uninfected), OcB-9 (7 infected and 7 uninfected), and OcB-43 (13 infected and 14 uninfected) were tested in 15 independent experiments. The age of mice was 8–23 weeks (mean = 11.9 weeks, median = 11 weeks) at the time of infection (start of experiment in control mice). A total of 81 infected mice of strains BALB/c ($n = 9$), STS ($n = 10$), CcS-5 ($n = 11$), O20 ($n = 12$), B10 ($n = 16$), B10.O20 ($n = 16$), and OcB-9 ($n = 7$) from these experiments were used for estimation of parasite load in skin and/or spleen. 40 infected female mice of the strains BALB/c ($n = 5$), STS ($n = 10$), CcS-5 ($n = 4$), CcS-16 ($n = 12$), and CcS-20 ($n = 9$) from additional four experiments were also used for the estimation of parasite load in skin and/or spleen.

Immunohistochemistry Experiments

97 (48 infected and 49 uninfected) female mice of strain BALB/c (9 infected and 9 uninfected), STS (9 infected and 9 uninfected), CcS-5 (8 infected and 8 uninfected), CcS-20 (11 infected and 11 uninfected), and O20 (11 infected and 12 uninfected) were tested in two independent experiments. The age of mice was 8–18 weeks (mean 13 weeks, median 14 weeks) at the time of infection.

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 (permissions Nr. 190/2010; 232/2012).

Parasite

Leishmania major LV 561 (MHOM/IL/67/LRCL 137 JERICHO II) was maintained in rump lesions of BALB/c females. Amastigotes were transformed to promastigotes using SNB-9 (35). 10^7 promastigotes from the passage two cultivated for 6 days were inoculated in 50 µl sterile saline s.c. into mouse rump (36). Control uninfected mice were injected by 50 µl sterile saline.

Disease Phenotype

The size of the skin lesions was measured every week using the Profi LCD Electronic Digital Caliper Messschieber Schieblehre Messer (Shenzhen Xtension Technology Co., Ltd. Guangdong, China), which has accuracy 0.02 mm. The mice were killed 8 weeks after inoculation. Skin, spleen, liver, and inguinal lymph nodes were collected for later analysis. Preparation of skin samples: approximately 3 mm of border skin surrounding lesion was taken. Hair was removed with scissors. A half of each skin sample was snap-frozen in liquid nitrogen for further RNA and DNA isolations. Another half was fixed in 4% formaldehyde for further paraffin embedding and immunohistochemical analysis. Samples from uninfected mice were obtained from the same rump area and used as a negative control.

Quantification of Parasite Load by PCR-ELISA

Parasite load was measured in DNA from frozen skin and spleen samples using PCR-ELISA according to the previously published protocol (37). Briefly, total DNA was isolated using a TRI reagent (Molecular Research Center, Cincinnati, OH, USA) standard procedure (https://www.mrcgene.com/wp-content/uploads/2014/06/TRI-LSMarch2017.pdf) or a modified proteinase K procedure (37). 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. major* DNA per reaction was amplified as a highest concentration of standard. A 33-cycle (expression experiments) or 26-cycle

(immunostaining experiments) PCR reaction was used for quantification of parasites. Under these conditions, the amount of PCR product is linearly proportional to number of parasites (37). PCR product was measured by the modified ELISA (Pharmingen, San Diego, CA, 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.

RNA Isolation

Mouse spleens, skins, liver, and inguinal lymph nodes were snapped frozen by liquid nitrogen immediately after dissection and stored at −80°C until total RNA extraction. At the time of RNA isolation tissue were homogenized in TRI Reagent (Sigma-Aldrich, Inc., St. Louis, MO, USA) using Polytron PT 2100 homogenizer (Kinematica Ag, Luzern, Switzerland) and immediately followed by total RNA isolation according to the manufacturer's protocol. RNA concentration was measured with Nanodrop (NanoDrop Technologies, LLC, Wilmington, DL), and quality of RNA was estimated also using Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). The isolated RNA was stored at −80°C.

Real-time PCR

One microgram of total RNA was diluted in 8 µl of sterile RNase- and DNase-free water, was treated with 1 µl DNase I (1 U/µl) and 1 µl DNase I reaction buffer (Promega Corporation, Madison, WI, USA), and used for subsequent reverse transcription. Single-strand cDNA was prepared from total RNA using Promega first-strand synthesis method. DNase I-treated RNA was incubated for 10 minutes at 65°C, then cooled quickly on ice for 5 minutes, and then treated with 1 µl DNase I stop solution (Promega Corporation, Madison, WI, USA). For the next step, a mixture containing 1 µl of random hexamers primers (100 ng/1 µl) (Invitrogen, Carlsbad, CA, USA), 5 µl (50 ng/µl) of dNTP mix (Invitrogen, Carlsbad, CA, USA), 5 µl of the reaction buffer (Promega Corporation, Madison, WI, USA), 2.5 µl of RNase/DNase-free water (Invitrogen, Carlsbad, CA, USA), and 0.5 µl of M-MLV Reverse Transcriptase RNAase H Minus Point Mutant (100 U/1 µl) (Promega Corporation, Madison, WI, USA) was added and followed by 60 minutes at 37°C. Single-strand cDNA was kept at −80°C until RT-PCR analysis. Real-time PCR was performed using a BioRad iQ iCycler Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primers were designed using Roche Universal ProbeLibrary, ProbeFinder version 2.45 for mouse (*Gbp2b*/*Gbp1*-F AAACCAGGAGGCTACTACCTTTTT, *Gbp2b*/*Gbp1*-R GTATTTTCTCAGCATCACTTCAGC; *Gbp5*-F TTCACCCAATCTAAGACCAAGAC, *Gbp5*-R AGCACCAG GCTTTCTAGACG; *Gapdh*-F AGAACATCATCCCTGCAT CC, *Gapdh*-R ACATTGGGGGTAGGAACAC). Reaction was performed in total volume of 25 µl, including 12.5 µl of 2× SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 1 µl of each primer of *Gbp2b*/*Gbp1* and *Gbp5* genes (final concentration 6.6 µM), 7.5 µl of water (Invitrogen, Carlsbad, CA, USA), and 3 µl of the cDNA template. Six different samples from each experimental group were used, and all samples were tested in triplets. The average Ct values (cycle threshold) were used for quantification, and the relative amount of each mRNA was normalized to the housekeeping gene, *Gapdh* mRNA. Using the delta Ct value, relative expression was calculated [ratio (reference/target) = 2 Ct (reference) − Ct (target)] × 10,000. All experiments included negative controls containing water instead of cDNA.

Genotyping of *Gbp* Cluster in OcB Series

DNA was isolated from tails using a standard proteinase procedure. Strains O20, B10, B10.O20, OcB-9, and OcB-43 were genotyped using microsatellite markers D3Mit160 (size of B10 allele 137 bp, size of O20 allele 127 bp) and D3Mit17 (B10 allele 200 bp, O20 allele 174 bp) (Generi Biotech, Hradec Králové, Czech Republic): The DNA genotyping by PCR was performed as described elsewhere (38).

Immunostaining

After deparaffinization and rehydration, the 3 µm thick slices of skin tissue were 15 minutes heat induced in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.5) for antigen retrieval. For fluorescent labeling of *Leishmania* parasite was used anti-*Leishmania* lipophosphoglycan mouse monoclonal antibody (cat. no. CLP003A; Cedarlane, Hornby, Canada) and TRITC-labeled IgM (115-025-020; Jackson ImmunoResearch, West Grove, PA) all diluted 1:500. Gbp2b/Gbp1 protein was stained with rabbit anti-Gbp1 Polyclonal antibody (PA5-23509; Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:100 and anti-rabbit-AlexaFluor-647 (cat. no. 711-605-152; Jackson ImmunoResearch, West Grove, PA) diluted 1:500. Nuclei of the cells were stained with bisBenzimide H33258 (Sigma-Aldrich, St. Louis, MO, USA) 10 mg per 1 ml diluted 1:1,000. Images were captured with microscope Leica DM6000 objective HCX PL Apo 40×/0.75 PH2 and color camera Leica DFC490. Evaluation of images was done with Fiji ImageJ 1.51n. 10 fields (320.66 × 239.57 µM) from each mouse were analyzed.

Statistical Analysis

The differences among strains within each of the two groups in expression of *Gbp2b*/*Gbp1* and *Gbp5* and the differences between uninfected and infected mice were evaluated by Mann–Whitney test using the program Statistica for Windows 12.0 (StatSoft, Inc., Tulsa, OK, USA). The results were corrected for multiple testing by Bonferroni correction. The correction factor was 10× both for intragroup differences and differences between infected and uninfected mice of the same strain.

RESULTS

Mouse Strains Differ in Expression of Both *Gbp2b*/*Gbp1* and *Gbp5* in Uninfected Mice

We observed strong genetic influence on mRNA levels of tested *Gbp*s. We have examined expression of *Gbp2b*/*Gbp1* (**Figure 1**) and *Gbp5* (**Figure 2**) in skin, inguinal lymph nodes, spleen, and liver of uninfected mice belonging to two genetically different series of strains—CcS/Dem (BALB/c, STS, CcS-5, CcS-16, CcS-20) and OcB/Dem (O20, B10, B10.O20, OcB-9, OcB-43). We have compared expression in parental strains BALB/c and STS with the

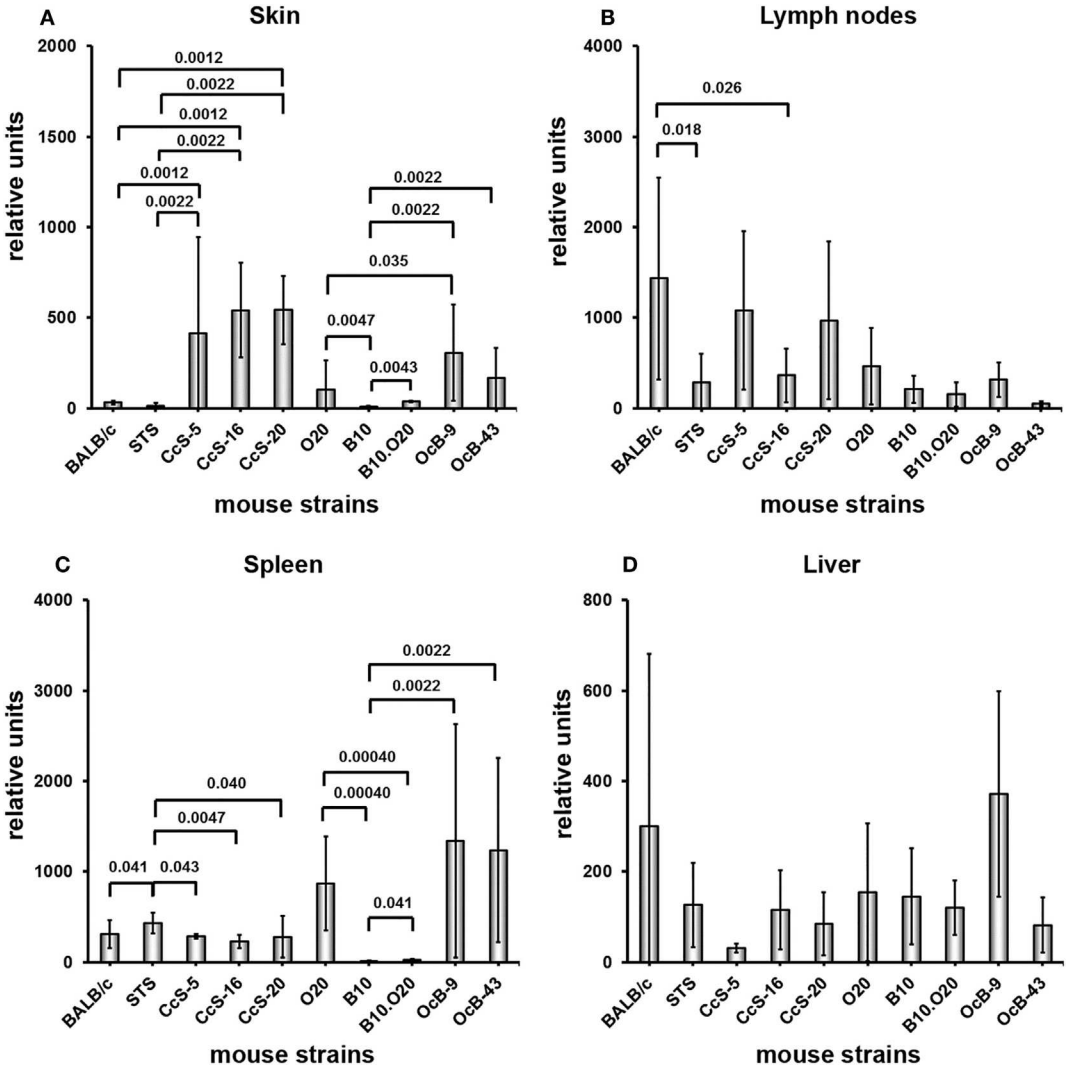

FIGURE 1 | Differences in expression of *Gbp2b/Gbp1* in organs of uninfected mice. Expression of *Gbp2b/Gbp1* in skin **(A)**, lymph nodes **(B)**, spleen **(C)**, and liver **(D)** of uninfected female mice of strains BALB/c (n = 7 skin, 9 lymph nodes, 11 spleen, 9 liver), STS (6 skin, 9 lymph nodes, 8 spleen, 6 liver), CcS-5 (6 skin, 6 lymph nodes, 6 spleen, 6 liver), CcS-16 (6 skin, 6 lymph nodes, 6 spleen, 6 liver), CcS-20 (6 skin, 6 lymph nodes, 7 spleen, 6 liver), O20 (7 skin, 6 lymph nodes, 9 spleen, 6 liver), B10 (6 skin, 7 lymph nodes, 6 spleen, 6 liver), B10.O20 (5 skin, 7 lymph nodes, 6 spleen, 8 liver), OcB-9 (6 skin, 6 lymph nodes, 6 spleen, 5 liver), and OcB-43 (6 skin, 6 lymph nodes, 6 spleen, 7 liver) were compared. The data show the means ± SD. Only the differences between parental strains BALB/c and STS and strains of CcS/Dem series and parental strains O20 and B10 and strains of OcB/Dem series are shown. Nominal P values are shown.

strains of CcS/Dem series, and expression in parental strains O20 and B10 with the strains of OcB/Dem series (**Figures 1** and **2**), as well as expression of the strains within CcS/Dem and OcB/Dem series in skin (**Tables 1A,C**), lymph nodes (**Tables 2A,C**), spleen (**Tables 3A,C**), and liver (**Tables 4A,C**).

Expression of *Gbp2b*/*Gbp1* in background strain BALB/c and donor strain STS in skin (**Figure 1A**; **Table 1A**) does not differ, whereas strains CcS-5, CcS-16, and CcS-20, each carrying a different set of 12.5% genes of STS on BALB/c background, exhibit higher expression than either parent (**Figure 1A**; **Table 1A**). Expression of *Gbp2b*/*Gbp1* in parental strains of OcB/Dem series O20 and B10 in skin differed ($P = 0.0047$); strains B10.O20, OcB-9, and OcB-43 exceeded in *Gbp2b*/*Gbp1* expression of the parental strain B10 but not O20 (**Figure 1A**; **Table 1A**).

We have observed differences in the expression of *Gbp2b*/*Gbp1* among mouse strains also in other tested organs (**Figures 1B–D**; **Tables 2A**, **3A**, and **4A**). Strains OcB-43 and OcB-9 differed in the expression of *Gbp2b*/*Gbp1* in lymph nodes (**Table 2A**), CcS-16 exhibited lower expression than STS in spleen (**Figure 2C**; **Table 3A**), B10 and B10.O20 exhibited lowest expression in spleen and differed from strains O20, OcB-9, and OcB-43, CcS-5 exhibited lower expression than CcS-16 in liver (**Figure 1D**; **Table 4A**); however, expression in none of the CcS or OcB strains exceeded the range of expression in both parental strains.

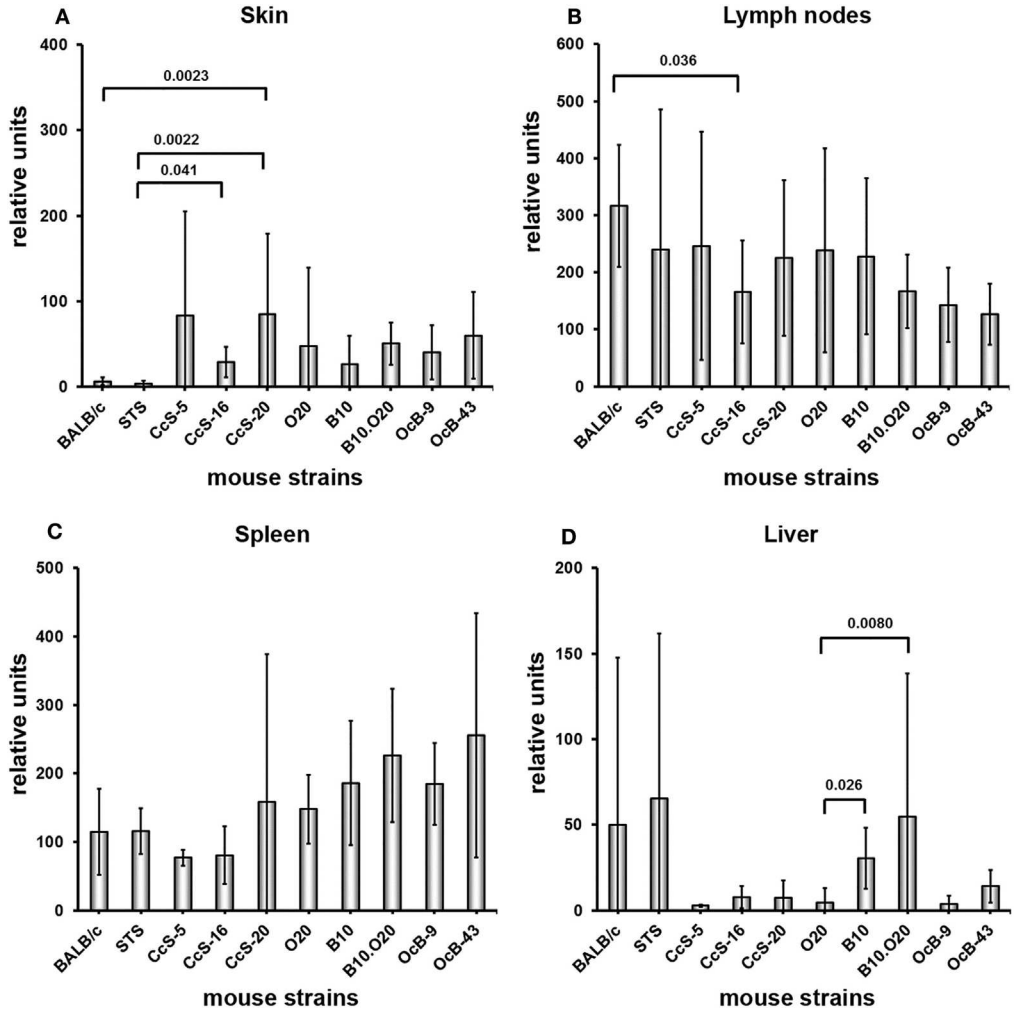

FIGURE 2 | Differences in expression of *Gbp5* in organs of uninfected mice. Expression of *Gbp5* in skin **(A)**, lymph nodes **(B)**, spleen **(C)** and liver **(D)** of uninfected female mice of strains of strains BALB/c (n = 7 skin, 9 lymph nodes, 11 spleen, 9 liver), STS (6 skin, 9 lymph nodes, 8 spleen, 6 liver), CcS-5 (6 skin, 6 lymph nodes, 6 spleen, 6 liver), CcS-16 (6 skin, 6 lymph nodes, 6 spleen, 6 liver), CcS-20 (6 skin, 6 lymph nodes, 7 spleen, 6 liver), O20 (7 skin, 6 lymph nodes, 9 spleen, 6 liver), B10 (6 skin, 7 lymph nodes, 6 spleen, 6 liver), B10.O20 (5 skin, 7 lymph nodes, 6 spleen, 8 liver), OcB-9 (6 skin, 6 lymph nodes, 6 spleen, 5 liver), and OcB-43 (6 skin, 6 lymph nodes, 6 spleen, 7 liver) were compared. The data show the means ± SD. Only the differences between parental strains BALB/c and STS and strains of CcS/Dem series and parental strains O20 and B10 and strains of OcB/Dem series are shown. Nominal P values are shown.

Expression of *Gbp5* in skin did not differ in parental strains of CcS/Dem series BALB/c and STS (**Figure 2A**; **Table 1C**), and expression of *Gbp5* in CcS-20 exceeded the expression of both parental strains (**Figure 2A**; **Table 1C**). There was no difference in *Gbp5* expression among strains of OcB/Dem series (**Table 1C**).

We did not find any significant differences in expression of *Gbp5* among the strains of both CcS/Dem and OcB/Dem series in the lymph nodes, spleen, and liver; none of the strains in CcS/Dem or in OcB/Dem series differed from either parent (**Figures 2B–D**; **Tables 2C**, **3C**, and **4C**).

Upregulation of *Gbp2b*/*Gbp1* and *Gbp5* mRNAs after Infection

In susceptible mice, pathology started as a nodule at the site of *L. major* infection appearing between weeks 2 and 4, which progressed in susceptible strains into a skin lesion (**Figure 3A**) (34). Strains BALB/c and CcS-16 are highly susceptible and develop large skin lesions (**Figures 3A,B**), B10.O20 develops moderate skin lesions (**Figures 3A,C**), CcS-20 is intermediate (**Figures 3A,D**) (34), and STS, CcS-5, O20, B10, OcB-9, and OcB-43 are resistant with no or minimal skin lesions (**Figure 3A**). All tested strains contain parasites in skin (**Figure 3D**) and spleen (**Figure 3E**), although the parasite load in resistant strains STS, CcS-5 and O20 (skin and spleen), and OcB-9 (spleen) is low.

Infection increased the expression of *Gbp2b*/*Gbp1* and *Gbp5* in organs of tested mice, the highest increase was observed in skin (**Figures 4**–**7**). All tested strains except CcS-5 and OcB-9 exhibited significantly higher expression of *Gbp2b*/*Gbp1* and *Gbp5* in skin after infection, irrespective of their susceptibility or resistance status (**Figure 4A**). Similarly as in uninfected mice,

TABLE 1 | Comparison of expression of *Gbp2b/Gbp1* or *Gbp5* among mouse strains of CcS/Dem and OcB/Dem series in skin.

CcS/Dem series					OcB/Dem series				
Strain	**BALB/c**	**STS**	**CcS-5**	**CcS-16**	**Strain**	**O20**	**B10**	**B10.O20**	**OcB-9**
A. *Gbp2b/Gbp1* uninfected									
BALB/c					**O20**				
STS	0.11				**B10**	0.0047			
CcS-5	0.0012	0.0022			**B10.O20**	1	0.0043		
CcS-16	0.0012	0.0022	0.24		**OcB-9**	0.035	0.0022	0.0043	
CcS-20	0.0012	0.0022	0.31	0.82	**OcB-43**	0.23	0.0022	0.052	0.18
B. *Gbp2b/Gbp1* infected									
BALB/c					**O20**				
STS	0.54				**B10**	0.043			
CcS-5	0.86	0.73			**B10.O20**	0.0012	0.19		
CcS-16	0.0076	0.0012	0.0022		**OcB-9**	0.54	0.40	0.0047	
CcS-20	0.0048	0.0012	0.0022	0.59	**OcB-43**	0.91	0.036	0.00012	0.30
C. *Gbp5* uninfected									
BALB/c					**O20**				
STS	0.37				**B10**	0.73			
CcS-5	0.30	0.13			**B10.O20**	0.20	0.25		
CcS-16	0.051	0.041	0.82		**OcB-9**	0.45	0.39	0.79	
CcS-20	0.0023	0.0022	0.31	0.093	**OcB-43**	0.14	0.18	0.79	0.59
D. *Gbp5* infected									
BALB/c					**O20**				
STS	0.54				**B10**	0.83			
CcS-5	0.18	0.37			**B10.O20**	0.21	0.076		
CcS-16	0.088	0.0012	0.0022		**OcB-9**	0.088	0.15	0.000026	
CcS-20	0.036	0.0023	0.0022	0.49	**OcB-43**	0.50	0.24	0.51	0.0012

A. Differences of Gbp2b/Gbp1 expression in uninfected skin; B. Differences of Gbp2b/Gbp1 expression in skin after 8 weeks of infection; C. Differences of Gbp5 expression in uninfected skin; D. Differences of Gbp5 expression in skin after 8 weeks of infection. Green: nominal (uncorrected) P value < 0.05; red: difference significant after correction for multiple testing at P < 0.05.

levels of *Gbp2b*/*Gbp1* mRNA in CcS-16 and CcS-20 exceeded those in both parental strains BALB/c and STS (Figure S1A in Supplementary Material; **Table 1B**); *Gbp5* expression in infected CcS-20 also exceeded that in both BALB/c and STS (Figure S2A in Supplementary Material; **Table 1D**).

Infection also induced increase of *Gbp2b*/*Gbp1* in inguinal lymph nodes of all strains except BALB/c and CcS-20, the highest expression was observed in CcS-5 (**Figure 5A**), which differed from all tested strains except STS (Figure S1B in Supplementary Material; **Table 2B**), but only increase of expression of B10.O20 and OcB-43 was significant after correction for multiple testing; we did not observe significant increase of *Gbp5* mRNA in lymph nodes (**Figure 5B**).

Four strains (BALB/c, STS, CcS-5, and CcS-16) show significantly increased expression of *Gbp2b*/*Gbp1* in spleen (**Figure 6A**). Expression of *Gbp5* was increased in CcS-5 (**Figure 6B**).

In liver, infection induced significant increases of *Gbp2b*/*Gbp1* mRNA in strains of CcS/Dem series, CcS-5, and CcS-16 (**Figure 7A**). Level of *Gbp2b*/*Gbp1* mRNA in CcS-16 is highest from all tested strains (Figure S1 in Supplementary Material; **Table 4B**). Expression of *Gbp5* was significantly increased in CcS/Dem strains CcS-5 and CcS-16 and decreased in OcB/Dem strain OcB-43 (**Figure 7B**; **Table 4D**).

Gbp2b/Gbp1 Protein Tends to Co-Localize with *Leishmania* Parasites in Skin of Resistant and Intermediate Strains but Not in the Highly Susceptible Strain BALB/c

Expression of *Gbp2b*/*Gbp1* mRNA was highest in skin of infected mice (Figure S1 in Supplementary Material; **Figure 4**), we have therefore analyzed by immunohistochemistry a presence of Gbp2b/Gbp1 protein in the skin of selected strains BALB/c, STS, CcS-5, CcS-20, and O20 and its relationship to *L. major* parasite in infected mice. **Figure 8** shows the presence of Gbp2b/Gbp1 protein in the skin of uninfected strains. The comparison of position of *L. major* and Gbp2b/Gbp1 in the skin of chronically infected highly susceptible strain BALB/c showed few Gbp2b/Gbp1 in the vicinity of *L. major* parasites, but a large part of parasites was free of Gbp2b/

TABLE 2 | Comparison of expression of *Gbp2b/Gbp1* or *Gbp5* among mouse strains of CcS/Dem and OcB/Dem series in inguinal lymph nodes.

CcS/Dem series					OcB/Dem series				
Strain	**BALB/c**	**STS**	**CcS-5**	**CcS-16**	**Strain**	**O20**	**B10**	**B10.O20**	**OcB-9**
A. *Gbp2b/Gbp1* uninfected									
BALB/c					**O20**				
STS	0.018				**B10**	0.53			
CcS-5	0.69	0.065			**B10.O20**	0.53	0.62		
CcS-16	0.026	0.59	0.18		**OcB-9**	0.70	0.37	0.051	
CcS-20	0.61	0.24	1	0.39	**OcB-43**	0.31	0.10	0.035	0.0022
B. *Gbp2b/Gbp1* infected									
BALB/c					**O20**				
STS	0.18				**B10**	0.021			
CcS-5	0.0049	0.13			**B10.O20**	0.038	0.28		
CcS-16	0.40	0.18	0.0022		**OcB-9**	0.30	0.081	0.13	
CcS-20	0.96	0.31	0.0087	0.49	**OcB-43**	0.15	0.25	0.019	0.64
C. *Gbp5* uninfected									
BALB/c					**O20**				
STS	0.39				**B10**	0.95			
CcS-5	0.61	0.82			**B10.O20**	0.37	0.62		
CcS-16	0.036	1	0.59		**OcB-9**	0.24	0.23	0.63	
CcS-20	0.22	1	1	0.39	**OcB-43**	0.18	0.051	0.30	0.59
D. *Gbp5* infected									
BALB/c					**O20**				
STS	0.96				**B10**	0.14			
CcS-5	0.53	0.59			**B10.O20**	0.37	0.69		
CcS-16	0.010	0.31	0.0022		**OcB-9**	0.92	0.24	0.48	
CcS-20	0.53	0.94	0.18	0.31	**OcB-43**	0.060	0.44	0.22	0.34

A. Differences of Gbp2b/Gbp1 expression in uninfected lymph nodes; B. Differences of Gbp2b/Gbp1 expression in lymph nodes after 8 weeks of infection; C. Differences of Gbp5 expression in uninfected lymph nodes; D. Differences of Gbp5 expression in lymph nodes after 8 weeks of infection. Green: nominal (uncorrected) P value < 0.05; red: difference significant after correction for multiple testing at P < 0.05.

Gbp1 (**Figure 9A**); the comparison of parasite load in the skin of the tested strains is shown in Figure S3 in Supplementary Material. In resistant strains STS (**Figure 9B**), CcS-5 (**Figure 9C**), and O20 (**Figure 9E**) and in intermediate strain CcS-20 (**Figure 9D**), Gbp2b/Gbp1 co-localized with clusters of parasites (**Figures 9B–E**) that in some places formed large clusters or long stretches. Gbp2b/Gbp1 either surrounded these clusters (**Figures 9B–D**) or formed stretches consisting of *L. major* parasites and Gbp2b/Gbp1 (**Figures 9C,E**). The tightest co-localization was observed in strains CcS-20 (**Figure 9D**) and O20 (**Figure 9E**).

DISCUSSION

Genetic Influence on Expression of *Gbp2b/Gbp1* and *Gbp5*

Tested strains exhibited genetic differences in *Gbps* expression both before and after *L. major* infection (**Figures 1**, **2**, **4** and **7**; **Tables 1–4**). Our study extends analysis of genetic influence by Staeheli and coworkers on Gbp2b/Gbp1 expression (39), who injected forty six mouse strains by poly(I);poly(C) in order to induce interferon production and tested their spleen cells for guanylate-binding activity. Tested strains were divided into Gbp2b/Gbp1 inducible and Gbp2b/Gbp1 noninducible groups. BALB/c was in the inducible group, whereas STS, O20, and C57BL/6J belonged to noninducible one (39). Our data confirm strong genetic influence on expression of *Gbp2b/Gbp1*; however, a direct comparison of outcome of study of Staeheli et al. (39) with our results is impossible due to different experimental designs. They induced Gbp2b/Gbp1 expression by poly(I);poly(C) that is structurally similar to double-stranded RNA present in some viruses, whereas we stimulated *Gbp2b/Gbp1* expression by the chronic infection with parasite *L. major*.

Comparison of Genotypes in *Gbp* Cluster on Mouse Chromosome 3 Indicates *Trans*-Regulation

Our data surprisingly showed that in several organs expressions levels of *Gbps* in recombinant congenic strains were outside the range of their parents. In skin of uninfected mice, expression

TABLE 3 | Comparison of expression of *Gbp2b/Gbp1* or *Gbp5* among mouse strains of CcS/Dem and OcB/Dem series in spleen.

CcS/Dem series					OcB/Dem series				
Strain	**BALB/c**	**STS**	**CcS-5**	**CcS-16**	**Strain**	**O20**	**B10**	**B10.O20**	**OcB-9**
A. *Gbp2b/Gbp1* uninfected									
BALB/c					**O20**				
STS	0.041				**B10**	0.00040			
CcS-5	0.66	0.043			**B10.O20**	0.00040	0.041		
CcS-16	0.40	0.0047	0.24		**OcB-9**	0.69	0.0022	0.0022	
CcS-20	0.25	0.040	0.23	0.84	**OcB-43**	0.53	0.0022	0.0022	0.94
B. *Gbp2b/Gbp1* infected									
BALB/c					**O20**				
STS	0.35				**B10**	0.000022			
CcS-5	0.40	0.018			**B10.O20**	0.00080	0.26		
CcS-16	0.44	0.018	0.49		**OcB-9**	0.53	0.00025	0.0022	
CcS-20	0.22	0.049	0.93	0.54	**OcB-43**	0.96	0.00025	0.0022	0.39
C. *Gbp5* uninfected									
BALB/c					**O20**				
STS	0.55				**B10**	0.46			
CcS-5	0.15	0.059			**B10.O20**	0.18	0.70		
CcS-16	0.30	0.14	1		**OcB-9**	0.27	1	0.49	
CcS-20	0.60	0.19	0.73	0.95	**OcB-43**	0.33	0.94	0.94	0.94
D. *Gbp5* infected									
BALB/c					**O20**				
STS	0.031				**B10**	0.32			
CcS-5	0.66	0.032			**B10.O20**	1	0.22		
CcS-16	0.08	0.00011	0.0022		**OcB-9**	0.61	0.26	0.59	
CcS-20	0.62	0.0061	0.18	0.247	**OcB-43**	0.61	0.26	0.94	0.24

A. Differences of Gbp2b/Gbp1 expression in uninfected spleen; B. Differences of Gbp2b/Gbp1 expression in spleen after 8 weeks of infection; C. Differences of Gbp5 expression in uninfected spleen; D. Differences of Gbp5 expression in spleen after 8 weeks of infection. Green: nominal (uncorrected) P value < 0.05; red: difference significant after correction for multiple testing at P < 0.05.

of *Gbp2b*/*Gbp1* in CcS-5, CcS-16, and CcS-20 exceeded those of both their parents BALB/c and STS (**Figure 1A**) and expression of *Gbp2b*/*Gbp1* in B10.O20 exceeded expression in parental strain B10 (**Figure 1A**). Such pattern of inheritance has been considered to be caused by *trans*-regulatory effects of non-linked or distant genes (40). The differences between parental strains and CcS/Dem strain CcS-20 persist after *L. major* infection, whereas the differences between expression of parents and CcS-5 and CcS-16 and between parent B10 and the strain B10.O20 disappear after infection (**Figure 1A**; Figure S1A in Supplementary Material; **Tables 1A,B**). Expression of *Gbp5* in skin of uninfected CcS-20 exceeded level of both parents (**Figure 2A**; **Table 1C**) but was significantly higher only than the parental strain STS after 8 weeks of infection (Figure S2A in Supplementary Material; **Table 1D**). CcS-5 and CcS-16 highly differed in the expression of both *Gbp1*/*Gbp2b* and *Gbp5* in lymph nodes and liver of infected mice; these strains also differed in expression of *Gbp5* in spleen (**Tables 2B,D**, **3D**, and **Tables 4B,D**).

Comparison of genotypes of the tested strains (33, 41, 42) (this study) in the *Gbp* cluster on the mouse chromosome 3 (**Figure 10**) revealed that strains CcS-5, CcS-16, and CcS-20 exhibiting higher expression of *Gbp* had *Gbp* genotype identical to that of BALB/c (C). Similarly, highly differing CcS-5 and CcS-16 strains carry the same *Gbp* allele. The presence of the same allele of *Gbp* gene cluster on chromosome 3 in strains that differ in other genes suggests that their differences in expression of *Gbp2*/*Gbp1* and/or *Gbp5* from one or both parents or from other RC strain are due to regulatory influence of non-*Gbp* gene(s) of STS origin carried on other genetic segments (*trans*-regulation). In the OcB/Dem series, B10.O20 carried in *Gbp* cluster B10 genotype (B), which similarly indicated *trans*-regulation of expression from O20 genome situated outside *Gbp* cluster (**Table 1A**; **Figure 10**). This *trans*-regulation can be partly overlaid by other regulatory events appearing after infection. Further genetic studies will be needed to elucidate nature of regulatory events observed in our studies.

The observations of progeny having a phenotype, which is beyond the range of the phenotype of its parents, are not rare. For example, analysis of gene expression from livers in chromosome substitution mouse strains revealed that only 438 of the 4,209 expressed genes were inside the parental range (40).

TABLE 4 | Comparison of expression of *Gbp2b/Gbp1* or *Gbp5* among mouse of CcS/Dem and OcB/Dem series strains in liver.

CcS/Dem series					OcB/Dem series				
Strain	**BALB/c**	**STS**	**CcS-5**	**CcS-16**	**Strain**	**O20**	**B10**	**B10.O20**	**OcB-9**
A. *Gbp2b/Gbp1* uninfected									
BALB/c					**O20**				
STS	0.46				**B10**	0.94			
CcS-5	0.088	0.24			**B10.O20**	0.85	0.66		
CcS-16	0.53	0.82	0.0022		**OcB-9**	0.052	0.13	0.045	
CcS-20	0.18	0.39	0.39	0.70	**OcB-43**	0.63	0.45	0.19	0.010
B. *Gbp2b/Gbp1* infected									
BALB/c					**O20**				
STS	0.072				**B10**	0.43			
CcS-5	0.21	0.31			**B10.O20**	0.66	0.98		
CcS-16	0.000074	0.0022	0.0022		**OcB-9**	0.054	0.0018	0.035	
CcS-20	0.0085	0.14	0.073	0.0012	**OcB-43**	0.49	0.042	0.40	0.014
C. *Gbp5* uninfected									
BALB/c					**O20**				
STS	0.33				**B10**	0.026			
CcS-5	0.86	0.18			**B10.O20**	0.0080	0.85		
CcS-16	0.46	0.94	0.041		**OcB-9**	0.79	0.052	0.011	
CcS-20	0.86	0.59	0.59	0.49	**OcB-43**	0.073	0.14	0.19	0.030
D. *Gbp5* infected									
BALB/c					**O20**				
STS	0.00030				**B10**	0.069			
CcS-5	0.0047	0.31			**B10.O20**	0.026	0.45		
CcS-16	0.77	0.0022	0.0022		**OcB-9**	0.78	0.54	0.33	
CcS-20	0.011	0.84	0.95	0.0023	**OcB-43**	0.23	0.13	0.062	0.95

A. Differences of Gbp2b/Gbp1 expression in uninfected liver; B. Differences of Gbp2b/Gbp1 expression in liver after 8 weeks of infection; C. Differences of Gbp5 expression in uninfected liver; D. Differences of Gbp5 expression in liver after 8 weeks of infection. Green: nominal (uncorrected) P value < 0.05; red: difference significant after correction for multiple testing at P < 0.05.

These observations are due to multiple regulatory interactions, which in new combinations of these genes in recombinant congenic or chromosomal substitution strains can lead to the appearance of new phenotypes that exceed their range in parental strains.

Increased Expression of *Gbp2b/Gbp1* and/or *Gbp5* in Resistant Mice Suggests Hidden Inflammation

We and others have demonstrated that *Leishmania* parasites are present not only in organs of infected susceptible mice with clinical manifestations of the disease but also in clinically asymptomatic mice of resistant strains (37, 43–46). This is also shown in **Figures 3D,E** and **9B,C,E**. **Figures 4–7** show that the expression of *Gbp2b/Gbp1* and/or *Gbp5* has increased after infection in at least one organ of each of the tested mice, including the resistant ones (STS, CcS-5, O20, B10, OcB-9, and OcB-43), which had no or only minimal and transient clinical symptoms. This strongly suggests that persistent parasites can contribute to the maintenance of protective immunity, which was manifested in our experiments by the increased levels of *Gbp2b/Gbp1* and *Gbp5* in resistant mice. It was demonstrated previously that this latent infection is controlled by inducible nitric oxide synthase (43) and phagocyte NADPH oxidase (46). It remains to be established, whether defense mechanisms including Gbps that were found to act against other pathogens (16, 23), operate also in *Leishmania*-infected mammalian host. In defense against *M. bovis*, Gbp2b/Gbp1 and Gbp7 could promote NADPH oxidase activity after the recruitment of gp91phox and gp22phox components to bacteria vacuoles (23), whereas parasite *T. gondii* was directly attacked via Gbp supramolecular complexes (16). The observed association (**Figure 9**) of Gbp2b/Gbp1 with *L. major* parasites in the skin of resistant and intermediate strains but not the highly susceptible strain BALB/c may suggest a role of this protein in response against the *L. major* pathogens.

Importantly, persistent parasites, besides stimulating protective immune reactions, can also represent a danger for hosts (45). The increased expression of *Gbp2b/Gbp1* and *Gbp5* in clinically asymptomatic mice reveals the price exacted from the

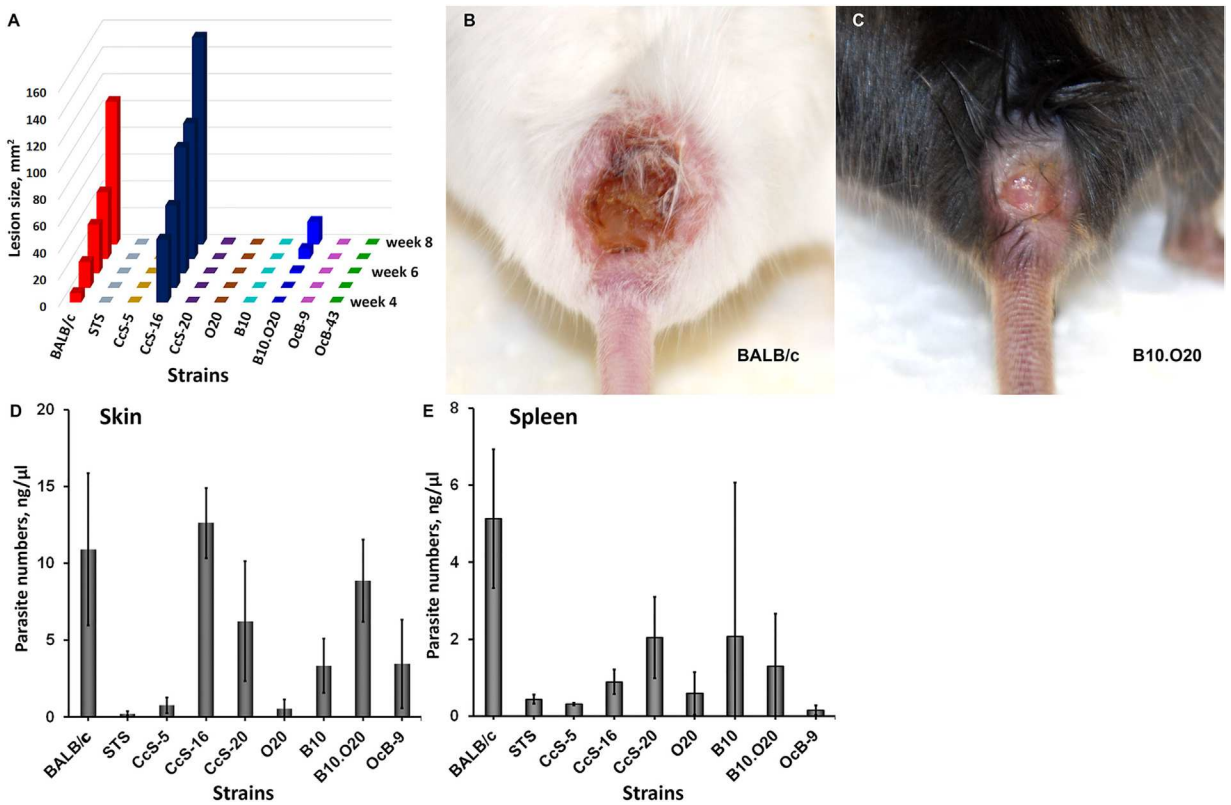

FIGURE 3 | Skin lesion development and parasite load in skin and spleen of infected mice. Kinetics of lesion development from week 4 (appearance of lesions in highly susceptible strains) till week 8 (end of experiment) **(A)**. Median values of skin lesions of mice tested in expression experiments are shown. Skin lesion caused by *Leishmania major* in female mouse of BALB/c strain at week 8 after infection **(B)**. Skin lesion caused by *L. major* in female mouse of B10.O20 strain at week 8 after infection **(C)**. Parasite load in skin **(D)** and spleen **(E)** of infected female mice of strains BALB/c (n = 5 skin, 11 spleen), STS (13 skin, 7 spleen), CcS-5 (11 skin, 4 spleen), CcS-16 (6 skin, 6 spleen), CcS-20 (7 skin, 9 spleen), O20 (6 skin, 12 spleen), B10 (9 skin, 13 spleen), B10.O20 (7 skin, 15 spleen), and OcB-9 (7 skin, 7 spleen). The data show the means ± SD.

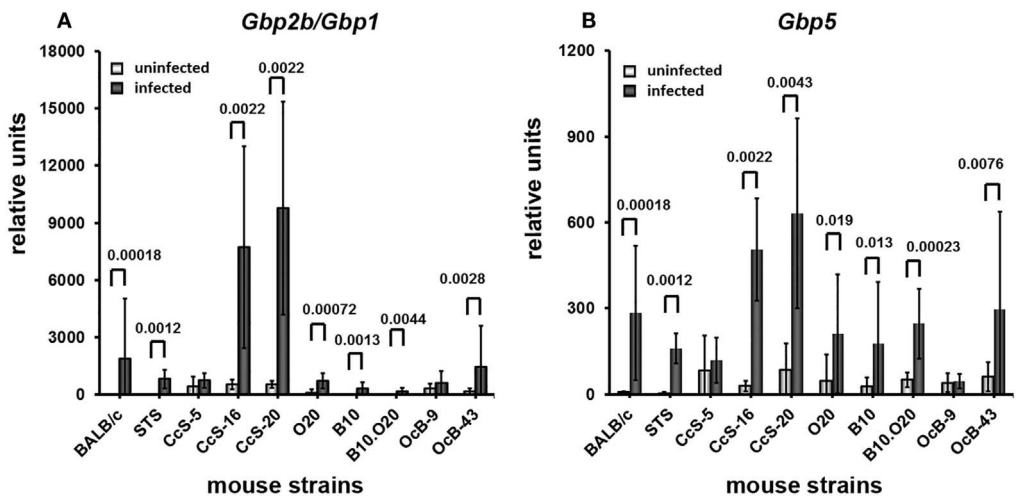

FIGURE 4 | Differences in expression of *Gbp2b/Gbp1* and *Gbp5* in skin of uninfected and infected mice. Expression of *Gbp2b/Gbp1* **(A)** and *Gbp5* **(B)** in skin of uninfected and infected female mice of strains BALB/c (9 infected and 7 uninfected), STS (7 infected and 6 uninfected), CcS-5 (6 infected and 6 uninfected), CcS-16 (6 infected and 6 uninfected), CcS-20 (6 infected and 6 uninfected), O20 (10 infected and 7 uninfected), B10 (8 infected and 6 uninfected), B10.O20 (13 infected and 5 uninfected), OcB-9 (7 infected and 6 uninfected), and OcB-43 (9 infected and 6 uninfected) were compared. Animals were subcutaneously inoculated with 10^7 promastigotes of *Leishmania major.* Control, uninfected mice were kept in the same animal facility. Both groups were killed in the same time—after 8 weeks of infection or start of experiment. The data show the means ± SD. Nominal P values are shown.

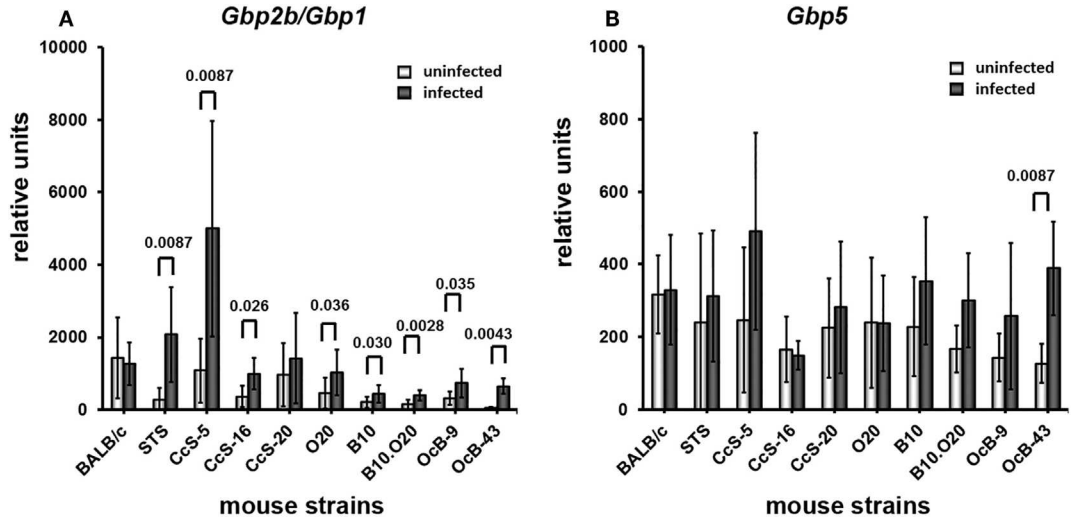

FIGURE 5 | Differences in expression of *Gbp2b/Gbp1* and *Gbp5* in inguinal lymph nodes of uninfected and infected mice. Expression of *Gbp2b/Gbp1* **(A)** and *Gbp5* **(B)** in inguinal lymph nodes of uninfected and infected female mice of strains BALB/c (11 infected and 9 uninfected), STS (6 infected and 6 uninfected), CcS-5 (6 infected and 6 uninfected), CcS-16 (6 infected and 6 uninfected), CcS-20 (6 infected and 6 uninfected), O20 (9 infected and 6 uninfected), B10 (13 infected and 7 uninfected), B10.O20 (11 infected and 7 uninfected), OcB-9 (7 infected and 6 uninfected), and OcB-43 (5 infected and 6 uninfected) were compared. Animals were subcutaneously inoculated with 10^7 promastigotes of *Leishmania major.* Control, uninfected mice were kept in the same animal facility. Both groups were killed after 8 weeks of infection. The data show the means ± SD from 12 independent experiments. Nominal *P* values are shown.

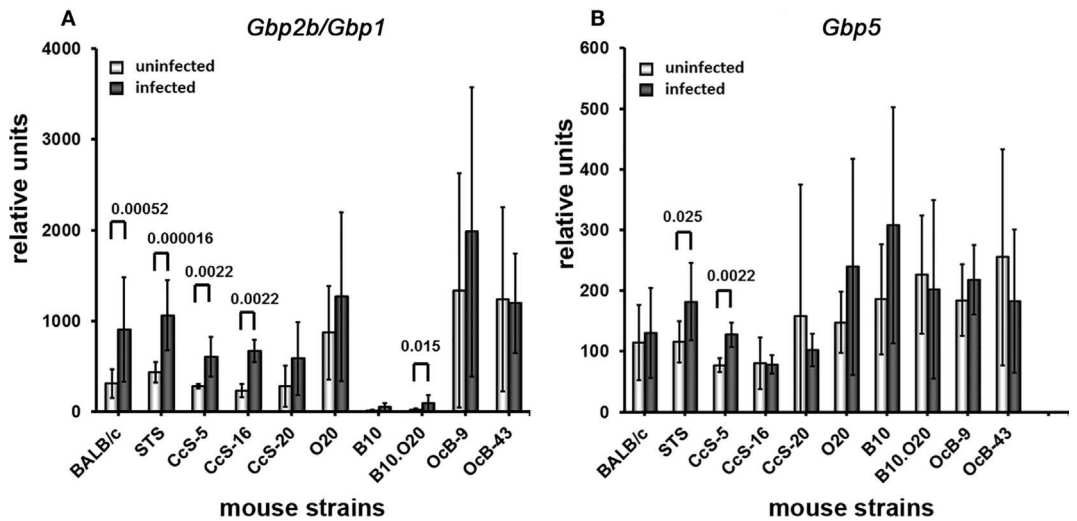

FIGURE 6 | Differences in expression of *Gbp2b/Gbp1* and *Gbp5* in spleen of uninfected and infected mice. Expression of *Gbp2b/Gbp1* **(A)** and *Gbp5* **(B)** in spleens of uninfected and infected female mice of strains BALB/c (14 infected and 11 uninfected), STS (12 infected and 8 uninfected), CcS-5 (6 infected and 6 uninfected), CcS-16 (6 infected and 6 uninfected), CcS-20 (5 infected and 7 uninfected), O20 (9 infected and 9 uninfected), B10 (10 infected and 6 uninfected), B10.O20 (6 infected and 6 uninfected), OcB-9 (6 infected and 6 uninfected), and OcB-43 (6 infected and 6 uninfected) were compared. Animals were subcutaneously inoculated with 10^7 promastigotes of *Leishmania major.* Control, uninfected mice were kept in the same animal facility. Both groups were killed after 8 weeks of infection. The data show the means ± SD from 12 independent experiments. Nominal *P* values are shown.

organism by a dormant infection. This finding deserves attention, because elevated levels of human GBP1 are directly involved in the endothelial dysfunction and the regulation of endothelial progenitor cells activity in patients with the autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and systemic sclerosis (47). In mice, elevated levels of Gbp3 and Gbp6 were linked with the pathogenesis of atherosclerosis (48). In humans with colorectal cancer, the anti-angiogenic effect of increased levels of GPB1 was beneficial in colorectal carcinoma patients, where it was associated with sustained reduction of intratumoral angiogenic activity and improved cancer-related survival (49).

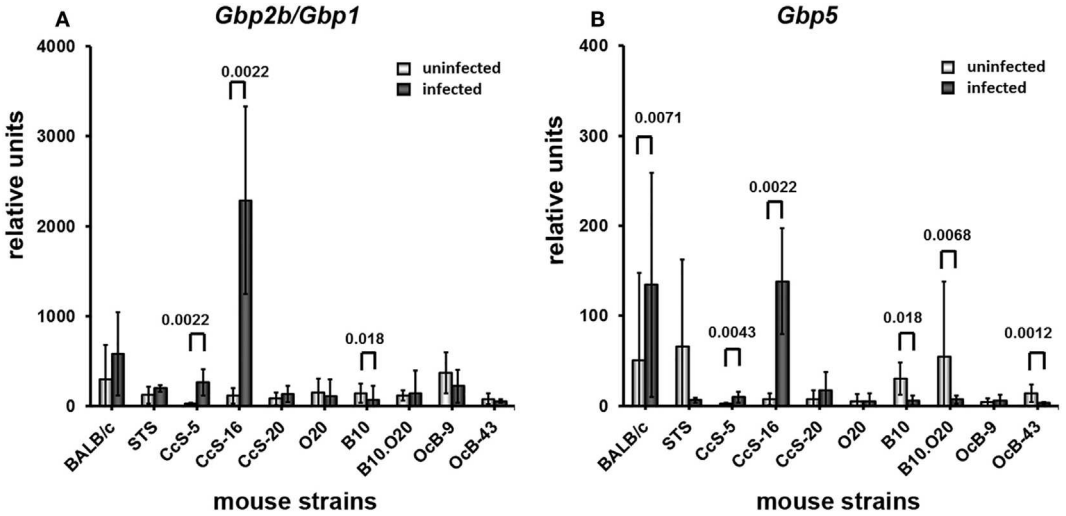

FIGURE 7 | Differences in expression of *Gbp2b/Gbp1* and *Gbp5* in liver of uninfected and infected mice. Expression of *Gbp2b/Gbp1* **(A)** and *Gbp5* **(B)** in liver uninfected and infected female mice of strains BALB/c (13 infected and 9 uninfected), STS (6 infected and 6 uninfected), CcS-5 (6 infected and 6 uninfected), CcS-16 (6 infected and 6 uninfected), CcS-20 (7 infected and 6 uninfected), O20 (8 infected and 6 uninfected), B10 (12 infected and 6 uninfected), B10.O20 (11 infected and 8 uninfected), OcB-9 (7 infected and 5 uninfected), and OcB-43 (7 infected and 6 uninfected) were compared. Animals were subcutaneously inoculated with 10^7 promastigotes of *Leishmania major.* Control, uninfected mice were kept in the same animal facility. Both groups were killed after 8 weeks of infection. The data show the means ± SD from 12 independent experiments. Nominal *P* values are shown.

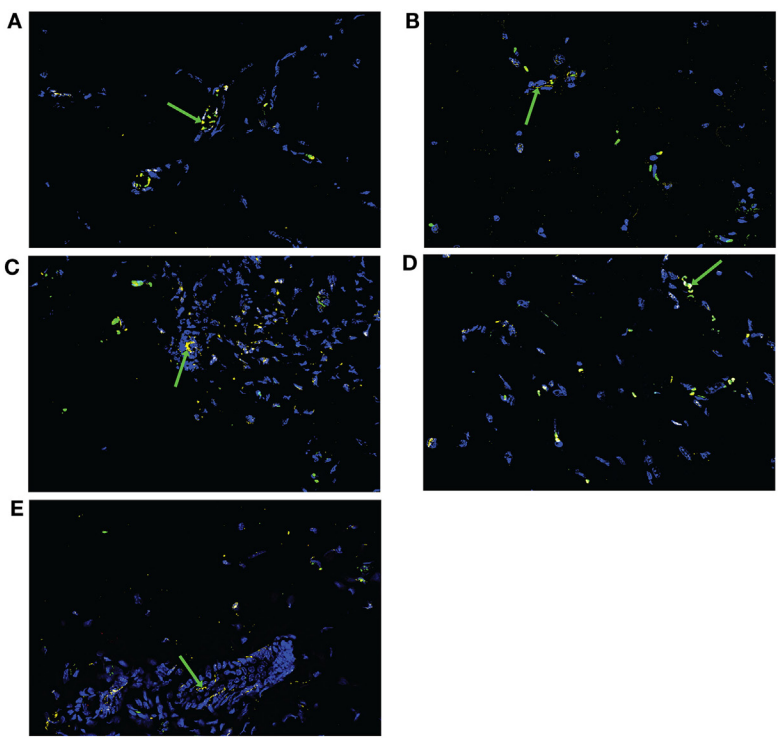

FIGURE 8 | Gbp2b/Gbp1 protein in skin of uninfected mice. Slices of skin tissue of females of BALB/c **(A)**, STS **(B)**, CcS-5 **(C)**, CcS-20 **(D)**, and O20 **(E)** mice were stained with the rabbit anti-Gbp1 Polyclonal antibody (PA5-23509, Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:100 and anti-rabbit-AlexaFluor-647 (cat. no. 711-605-152; Jackson ImmunoResearch, West Grove, PA) diluted 1:500. Nuclei of the cells were stained with bisBenzimide H33258 (Sigma-Aldrich, St. Louis, MO, USA) 10 mg per 1 ml diluted 1:1,000. Images were captured with microscope Leica DM6000 objective HCX PL Apo 40x/0.75 PH2 and color camera Leica DFC490. Evaluation of images was done with Fiji ImageJ 1.51n. Figures are representatives of data from 8 to 12 mice (see Materials and Methods) in 3 of them 10 fields (320.66 × 239.57 μM) from each mouse were analyzed, in the rest one field was analyzed to verify the results. Green arrows show Gbp2b/Gbp1 protein (yellow color), cell nuclei are stained in blue.

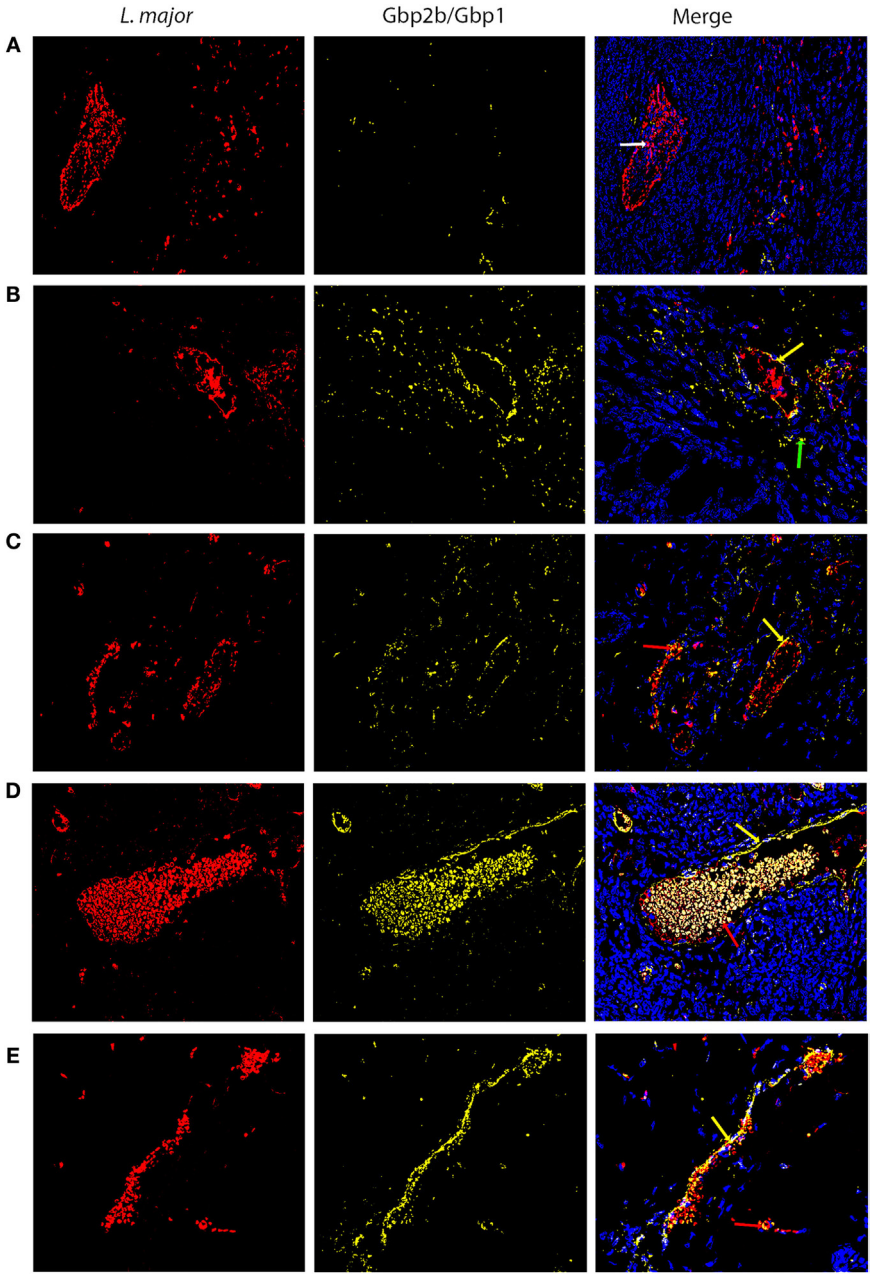

FIGURE 9 | Gbp2b/Gbp1 protein and *Leishmania major* parasites in skin of infected mice. Slices of skin tissue of females of BALB/c **(A)**, STS **(B)**, CcS-5 **(C)**, CcS-20 **(D)**, and O20 **(E)** mice infected for 8 weeks with *L. major* were stained with the anti-*Leishmania* lipophosphoglycan mouse monoclonal antibody (cat. no. CLP003A, Cedarlane, Hornby, Canada) and TRITC labeled IgM (115-025-020, Jackson ImmunoResearch, West Grove, PA) all diluted 1:500 and the rabbit anti-Gbp1 Polyclonal antibody (PA5-23509, Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:100 and anti-rabbit-AlexaFluor-647 (cat. no. 711-605-152; Jackson ImmunoResearch, West Grove, PA) diluted 1:500. Nuclei of the cells were stained with bisBenzimide H33258 (Sigma-Aldrich, St. Louis, MO, USA) 10 mg per 1 ml diluted 1:1,000. Images were captured with microscope Leica DM6000 objective HCX PL Apo 40×/0.75 PH2 and color camera Leica DFC490. Evaluation of images was done with Fiji ImageJ 1.51n. Figures are representatives of data from 8 to 11 mice (see Materials and Methods) in 3 of them 10 fields (320.66 × 239.57 μM) from each mouse were analyzed, in the rest one field was analyzed to verify the results. White arrow shows *L. major* amastigotes (red color), green arrows show Gbp2b/Gbp1 protein (yellow color), red arrows point to amastigotes co-localized with Gbp2b/Gbp1, whereas yellow arrows show either Gbp2b/Gbp1 surrounding parasite clusters or stretch of parasites and Gbp2b/Gbp1. Cell nuclei are stained in blue.

The immune reactions accompanying persistent *Leishmania* infection might be very important, because in addition to 12 million people presently suffering from the clinical manifestations of leishmaniasis (50), there are at least 120 million people with asymptomatic infection (45). It needs to be elucidated, whether such clinically asymptomatic people harboring persistent *Leishmania* parasites are more prone to immune-related diseases.

Strain	origin of *Gbp* cluster
BALB/c	C
STS	S
CcS-5	C
CcS-16	C
CcS-20	C
O20	O
B10	B
B10.O20	B
OcB-9	O
OcB-43	O

FIGURE 10 | Genetic origin (alleles) of *Gbp* cluster on chromosome 3 of tested strains. C—genotype of BALB/c origin, S—genotype of STS origin, O—genotype of O20 origin, B—genotype of B10 origin.

CONCLUSION

Our results represent the presently most comprehensive information about expression of *Gbps* in leishmaniasis *in vivo*.

We found that expression of *Gbp2b*/*Gbp1* and *Gbp5* is under strong genetic control involving in some strains also *trans*-regulation both in uninfected and *L. major*-infected mice.

We have observed that in several organs, expression of *Gbps* in recombinant congenic strains was outside the range of their parents. Tests of different strains that carry the same *Gbp* cluster genotypes on chromosome 3 indicate a *trans*-regulation of *Gbp2b*/*Gbp1* and *Gbp5* by genes that are not closely linked to *Gbp* genes. This finding may open way to identification and manipulation of these presently unknown genes.

Our results also point out that expression of *Gbp2b*/*Gbp1* and *Gbp5* was increased even in organs of resistant mice, which might suggest a hidden inflammation. It remains to be established whether the clinically asymptomatic infection might represent danger in predisposing organism to other diseases.

Co-localization of Gbp2b/Gbp1 protein with most *L. major* parasites in skin of resistant and intermediate strains STS, CcS-5, O20, and CcS-20 but not in highly susceptible BALB/c mice suggests that this molecule might play role in defense against leishmaniasis and opens new research direction in analysis of control of persistent parasites.

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 (permissions Nr. 190/2010; 232/2012).

AUTHOR CONTRIBUTIONS

YS planned and performed parasitology and expression experiments and contributed to the writing of the manuscript. VV performed parasitology experiments, analyzed the data, and contributed to the writing of the manuscript. TK contributed to the writing of the manuscript, parasitology experiments, estimation of parasite numbers, and data analysis. HH designed and performed immunohistochemistry analysis and analyzed the data. IK contributed to the estimation of parasite numbers. MS performed parasitology experiments. PD analyzed the data and contributed to the writing of the manuscript. ML conceived the study, interpreted data, and wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00130/full#supplementary-material.

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Supplements 2

Genetic regulation of Fcγ receptor IV in leishmaniasis

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ABSTRACT

Fcγ receptor IV (FCGR4), the receptor for the Fc fragment of immunoglobulin G (IgG), participates in IgG2a- and IgG2b-dependent effector functions in autoimmune responses and antibody-dependent cellular cytotoxicity in mouse. Experiments with mice bearing knockouts of other Fcγ receptors have shown that the genetic background of the host controls their role in response to *Leishmania* parasites, leading either to protective immunity or to progression of disease. There is also genetic evidence that human gene *FCGR2A* might play a role in IgG-mediated inflammation in asymptomatic *L. infantum* infection. However, the information about the role of FCGR4 in leishmaniasis is missing. We therefore studied levels of *Fcgr4* mRNA in skin, inguinal lymph nodes, spleen and liver after *L. major* infection and in uninfected controls. We tested two genetic series of mouse strains: BALB/c, STS and CcS-5, CcS-16 and CcS-20 that consist of different combinations of BALB/c and STS genomes, and strains O20, C57BL/10 (B10) and B10.O20, OcB-9 and OcB-43 carrying different mixtures of O20 and B10 genomes. BALB/c and CcS-16 are highly susceptible, B10.O20 is susceptible, CcS-20 intermediate and strains STS, CcS-5, O20, B10, OcB-9, OcB-43 are resistant with no clinical manifestations of the disease despite the presence of parasites in their organs.

We observed strong genetic influence on *Fcgr4* mRNA levels. Some strains differed in *Fcgr4* expression prior to infection. Several of them differed in *Fcgr4* expression although they carried the same *Fcgr4* allele, indicating its trans-regulation. Trans-regulation was observed also in some infected strains.

Infection caused a varying degree of up-regulation (up to 50x) of *Fcgr4* in all tested organs of mouse strains irrespective of their susceptibility or resistance. It was most pronounced in skin, where all strains except OcB-9 exhibited increase of *Fcgr4* mRNA. The up-regulation of FCGR4 after infection was confirmed by immunohistochemistry. We have compared localization of the parasites and FCGR4 in skin and liver of selected strains and found their partial co-localization. These findings suggest relationship of this molecule to the response to *L. major* infection.

Key words: *Fcgr4*, *Leishmania major*, recombinant congenic strains, clinically asymptomatic disease, inflammation, genetic regulation, trans-regulation, co-localization with parasites

INTRODUCTION

IgG antibodies participate in defense of the organism against pathogens. They can neutralize infectious agents and their products by inhibiting parts of a pathogen that are essential for its invasion and survival, by activating classical complement pathway and by binding to Fcγ receptors (FcγRs) on the membrane of immune cells. Depending on the cell and FcγR type, crosslinking of FcγRs on the cell surface activates several effector functions including phagocytosis, stimulation of respiratory burst, cell degranulation, antibody-dependent-cell-mediated cytotoxicity, and expression of chemokines and cytokines (1, 2). Depending on their functions, there are two main groups of the FcγRs: activating and inhibitory receptors. Activating FcγRs either have an immunoreceptor tyrosine-based activation motif (ITAM) in their intracytoplasmic domain or associate with the ITAM-containing signaling subunit FcR common γ chain, while inhibitory receptors carry an immunoreceptor tyrosine-based inhibition motif (ITIM). FcγRs are also classified depending on their affinity for IgGs. A few receptors can bind to monomeric IgG, which is definition of high-affinity receptors, while the other receptors predominantly bind to aggregated IgGs (3).

Mouse Fcγ receptor IV (FCGR4) is a glycoprotein that consists from the α chain with two extracellular globular domains and the dimer of the FcR common γ chain and its expression is restricted to myeloid-derived cell lineages: monocytes/macrophages, neutrophils and monocyte-derived dendritic cells (3, 4, 5). FcγRIV is encoded by the gene *Fcgr4* (also called *Fcgr3a*, *Fcrl3* and *CD16-2*) that is situated on the distal part (171.02 Mbp; 78.53 cM) of the mouse chromosome 1 in the *Fcgr* cluster between the genes *Fcgr2b* and *Fcgr3* (6). FcγRIV binds to the mouse IgG2a and IgG2b isotypes (6, 7) and participates in IgG2a- and IgG2b-dependent effector functions in autoimmune responses and antibody-dependent cell-mediated cytotoxicity. FcγRIV can also interact with IgE, which is involved in phagocytosis and inflammatory response by macrophages (8). This activation is restricted to mouse strains that carry IgE^b allele of the heavy chain of IgE (*Igh-*

7) (8) present in the strains C57BL/6, SJL/J, C57BL/10A, but not in the strains BALB/c, C3H/HeN, C3H.SW/Hz, CBA/J, A/J and AKR/J (9). *Fcgr4* exhibits 63% sequence identity to the human *FCGR3A* (Fc fragment of IgG receptor IIIa, CD16A)(6), which is localized at 1q23.3 on the long (q) arm of chromosome 1. As mouse FcγRIV can also bind IgE (see above), it has been believed to function similarly as the human IgE receptor FcεRI (10).

Due to diverse biological functions of FcγRs, these molecules are involved in susceptibility to cancer, autoimmune, allergic and infectious diseases (11, 12), including leishmaniasis, a disease caused by protozoan parasites *Leishmania* ssp. transmitted to mammalian host by the bite of infected *Phlebotomine* sand flies (13). More than 12 million people currently suffer by clinical manifestations of the disease, which occurs in three main forms: cutaneous, mucocutaneous and visceral leishmaniasis (14). It is assumed that there are at least 120 million infected individuals without the clinical signs of the disease (15). *Leishmania* parasites infect professional phagocytes (macrophages, monocytes and neutrophils), and also dendritic cells and fibroblasts (16). Internalization of *Leishmania* parasites into these cells is mediated by complement receptors CR1 (CD35) and CR3 (CD11b/CD18), mannose-fucose receptor (CD206), fibronectin receptors (CD49d.CD29, CD49.e/CD29, CD41/CD61), and several FcγRs (CD64, CD32, CD16) and DC-SIGN (CD209) (17). Thus, FcγRs are directly involved in *Leishmania* infection. Their influence on susceptibility to the disease depends on genotype of the host (18, 19), on parasite species (19, 20) and likely also on the experimental design (18, 19). BALB/c mice with deleted FcγR common chain exhibited enhanced resistance to *L. major* manifested by smaller skin lesions containing less parasites than wild type BALB/c (18), whereas an opposite outcome – increased susceptibility - was observed with FcγR knockouts on C57BL/6 genetic background (19). On the other hand, FcγR knockouts on C57BL/6 background were more resistant to *L. mexicana* (20) than wild type mice. It needs to be mentioned that the described results are not completely comparable, because in experiments leading to increased susceptibility of FcγR knockouts, parasites were injected

intradermally into ear (19), whereas in the experiments resulting in increased resistance of the knockouts the parasites were inoculated into hind footpad (18, 20). In human family-based study, polymorphism in *FCGR2A* (*CD32A*) was associated with delayed-type hypersensitivity (DTH)(Montenegro test) after infection with *L. infantum* (21). However, nothing is known about the role of FCGR4 in leishmaniasis.

We therefore analyzed FCGR4 in leishmaniasis using a highly sensitive system of recombinant congenic strains with high power to detect genetic differences in biological functions, which might escape detection using conventional inbred strains. We studied expression of *Fcgr4* mRNA in skin, inguinal lymph nodes, spleen and liver after *L. major* infection and in uninfected controls using two different genetic groups of mouse strains: the BALB/c-c-STS/Dem (CcS/Dem) and O20/A-c-C57BL/10-$H2^{pz}$ (OcB/Dem) series. Recombinant congenic strains of the CcS/Dem series CcS-5, CcS-16 and CcS-20 contain a different, random set of approximately 12.5% genes of the donor strain STS/A (STS) and approximately 87.5% genes of the background strain BALB/cHeA (BALB/c) (22, 23). The donor strain of the OcB/Dem series C57BL/10-$H2^{pz}$ (B10.O20) is a *H2* congenic strain on the C57BL/10Sn (B10) background (N8), which carries $H2^{pz}$ haplotype derived from the strain O20/A (O20) (23). The congenicity of the strain B10.O20 is however incomplete, in addition to *H2* region, approximately 1.68% of its genome is derived from the strain O20. The OcB/Dem strains OcB-9 and OcB-43 contain 12.5% or 6.25%, respectively, of non-*H2* genes of the B10 strain spread in the O20 genome (23). We also tested presence of FCGR4 protein in skin and liver in some of these strains.

Strains BALB/c and CcS-16 are highly susceptible to *L. major* and develop large skin lesions (24, 25) (Figure 1 A,B) as well as extensive splenomegaly and hepatomegaly (24), B10.O20 is susceptible (Figure 1 A,C), CcS-20 shows intermediate pathology and strains STS, CcS-5, O20, B10, OcB-9, OcB-43 are resistant and do not exhibit signs of clinical manifestations of the disease despite the presence of parasites in their organs (Figure 1D,E)(24, 25, 26).

Materials and Methods

Mice

A total of 275 (152 infected and 123 uninfected) female mice of strains BALB/c (33 infected and 22 uninfected), STS (20 infected and 13 uninfected), CcS-5 (11 infected and 10 uninfected), CcS-16 (10 infected and 11 uninfected), CcS-20 (12 infected and 12 uninfected), O20 (12 infected and 12 uninfected), B10 (17 infected and 10 uninfected), B10.O20 (17 infected and 12 uninfected), OcB-9 (7 infected and 7 uninfected) and OcB-43 (13 infected and 14 uninfected) were tested in 15 independent experiments. Age of mice was 8 to 23 weeks (mean = 11.9 weeks, median = 11 weeks) at the time of infection (start of experiment in control mice). The same mice were used to study expression of *Gbp2b* and *Gbp5* mRNA in experiments described in Sohrabi and coworkers (25).

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 (permissions Nr. 190/2010; 232/2012).

Parasite

Leishmania major LV 561 (MHOM/IL/67/LRCL 137 JERICHO II) was maintained in rump lesions of BALB/c females. Amastigotes were transformed to promastigotes using SNB-9 (27). 10^7 promastigotes from the passage two cultivated for six days were inoculated in 50 μl phosphate buffered saline s.c. into mouse rump (28). Control uninfected mice were injected by 50 μl sterile saline.

Disease phenotype

The size of the skin lesions was measured every week using the Profi LCD Electronic Digital Caliper Messschieber Schieblehre Messer (Shenzhen Xtension Technology Co., Ltd. Guangdong, China), which has accuracy 0.02 mm. The mice were sacrificed 8 weeks after inoculation. Skin, spleen, liver and inguinal lymph nodes were collected for later analysis. Preparation of skin samples: Approximately 3 mm of border skin surrounding lesion was taken. Hair was removed with scissors. A half of each skin sample was snap-frozen in liquid nitrogen for later RNA isolation. Another half was frozen embedded in O.C.T. (Sakura Finetek, Inc. USA) for further immunohistochemical (IHC) analysis. Samples from uninfected mice were obtained from the same rump area and used as a negative control (25).

RNA isolation

Mouse spleens, skins, liver and inguinal lymph nodes were snapped frozen by liquid nitrogen immediately after dissection and stored at -80˚C until total RNA extraction. RNA was extracted as described elsewhere (25) and stored at -80˚C.

Real-time PCR

The reaction was performed as described in (25). One µg of total RNA was diluted in 8 µl of sterile RNase and DNase free water and were treated with 1 µl DNase I (1U/ µl) and 1 µl DNase I reaction buffer (Promega Corporation, Madison, WI) and used for subsequent reverse transcription. Single strand cDNA was prepared from total RNA using Promega first-strand synthesis method. DNase I treated RNA was incubated for 10 min at 65 ◦C and then cooled quickly on ice for 5 min and then treated with 1 µl DNase I stop solution (Promega Corporation, Madison, WI). For the next step a mixture containing 1 µl of random hexamers primers (100 ng/ 1µl) (Invitrogen, Carlsbad, CA), 5 µl (50 ng/µl) dNTP mix (Invitrogen, Carlsbad, CA), 5µl of the reaction buffer (Promega Corporation, Madison, WI), 2.5 µl of RNase/ DNase free water (Invitrogen, Carlsbad, CA) and 0.5 µl of M-MLV Reverse Transcriptase RNAase H Minus Point Mutant (100 U/1 µl) (Promega Corporation, Madison,

WI) was added and followed by 60 min at 37°C. Single strand cDNA was kept at – 80 °C until RT PCR analysis. Real-time PCR was performed using a BioRad iQ iCycler Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). Primers were designed using Roche Universal ProbeLibrary, ProbeFinder version 2.45 for mouse: *Fcgr4-F* GGGCTCATTGGACACAACA, *Fcgr4-R* ATGGATGGAGACCCTGGAT. *Gapdh*-F AGAACATCATCCCTGCATCC, *Gapdh*-R ACATTGGGGGTAGGAACAC. Reaction was performed in total volume of 25 µl, including 12.5 µl 2x SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA), 1 µl of each primer of *Fcgr4* gene (final concentration 6.6 µM), 7.5 µl water (Invitrogen, Carlsbad, CA), and 3 µl of the cDNA template. At least six different samples from each experimental group were used, and all samples were tested in triplets. The average Ct values (Cycle threshold) were used for quantification and the relative amount of each mRNA was normalized to the housekeeping gene, *Gapdh* mRNA. Using the delta Ct value, relative expression was calculated (Ratio (reference/target) = 2 Ct (reference)-Ct (target)) x 10000. All experiments included negative controls containing water instead of cDNA.

Genotyping of *Fcgr4* in OcB/Dem series

DNA was isolated from tails using a standard proteinase K procedure. Strains O20, B10, B10.O20, OcB-9 and OcB-43 were genotyped using microsatellite markers D1Mit205 (169.2 Mbp, 76.43 cM), D1Mit205 (170.4 Mbp, 76.84 cM) and D1Mit456 (172.6 Mbp, 79.96 cM) (Generi Biotech, Hradec Králové, Czech Republic): The DNA genotyping by PCR was performed as described elsewhere (29, 30).

Immunostaining

For IHC we used frozen tissue embedded in O.C.T. (Sakura Finetek, Inc. USA). 10 µm slices were stained with the anti-*Leishmania* lipophosphoglycan mouse monoclonal antibody (cat. no. CLP003A, Cedarlane, Hornby, Canada) and TRITC labeled IgM (115-025-020, Jackson ImmunoResearch, West Grove, PA) all diluted 1:500, the rabbit anti- CD16-2/FcgRIV monoclonal antibody (50036-R012, Sino Biological, Beijing, China) diluted 1:1,000 and anti-rabbit-AlexaFluor-448 (111-545-003;

Jackson ImmunoResearch, West Grove, PA) diluted 1:1,000 or anti-rabbit-AlexaFluor-647 (711-605-152; Jackson ImmunoResearch, West Grove, PA) diluted 1:1,000, rat F4/80 monoclonal antibody conjugated with AlexaFluor-647 (51-4801-80, eBioscience, Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:100, rat Ly6G (GR1) monoclonal antibody (14-5931-82 Invitrogene, Thermo Fisher Scientific, Rockford, IL, USA) diluted 1:1,000 and anti-rat-AlexaFluor-488 (112-545-003, Jackson ImmunoResearch, West Grove, PA) diluted 1.1,000. Nuclei of the cells were stained with bisBenzimide H33258 (Sigma-Aldrich, St. Louis, MO, USA) 10 mg per 1 ml diluted 1:1,000. Samples were captured with DM6000 objective HCX PL Apo 40×/0.75 PH2 and color camera Leica DFC490. Evaluation of images was done with Fiji ImageJ 1.51n.

Statistical Analysis

The intragroup strain differences in expression of *Fcgr4* within the group of BALB/c-STS-derived strains and within the group of O20-B10-derived strains as well as differences between uninfected and infected mice were evaluated by Mann-Whitney test using the program Statistica for Windows 12.0 (StatSoft, Inc., Tulsa, OK). The results were corrected for multiple testing by Bonferroni correction. The correction factor was 10x both for intragroup differences and differences between infected and uninfected mice of the same strain.

RESULTS

We analyzed three types of differences: i) among uninfected mice of different strains, ii) between uninfected and infected mice of the same strain, and iii) among infected strains of different strains.

Strong genetic influence on expression of *Fcgr4* mRNA in uninfected mice

Analysis of expression of *Fcgr4* in skin, inguinal lymph nodes, spleen and liver of uninfected mice within two genetically different series of mouse strains: CcS/Dem (BALB/c, STS, CcS-5, CcS-16, CcS-20) and OcB/Dem (O20, B10, B10.O20, OcB-9, OcB-43) revealed strong genetic influence on

mRNA level **(Figure 2**, **Table 1 A, Table 2 A, Table 3 A, Table 4 A)** in every tested organ in at least one of the tested series. We compared expression of *Fcgr4* in parental strains BALB/c and STS with the strains of CcS/Dem series, and expression in parental strains O20 and B10 with the strains of OcB/Dem series (**Figure 2)**, as well as expression of the strains within CcS/Dem and OcB/Dem series in skin (**Table 1 A**), lymph nodes **(Table 2 A)**, spleen **(Table 3 A)** and liver **(Table 4 A**). In order to establish whether the differences in *Fcgr4* mRNA expression are due to cis- or trans-regulation, we determined genotypes at segment on the distal part on chromosome 1 carrying *Fcgr4* using either already published data (23, 31) or DNA typing (see Materials and Methods). All tested strain (CcS-5, CcS-16 and CcS-20) of the CcS/Dem series carry a BALB/c-derived *Fcgr4* allele, in the OcB/Dem series the strain B10.O20 carries B10-derived *Fcgr4* allele, whereas strains OcB-9 and OcB-43 carry an O20-derived *Fcgr4* allele **(Table 1)**.

Skin: Expression of *Fcgr4* in background parental strain BALB/c and donor parental strain STS in skin **(Figure 2 A, Table 1 A)** does not differ, whereas strains CcS-5 and CcS-20, each carrying a BALB/c-derived *Fcgr4* allele, exhibit higher expression than BALB/c, and CcS-20 differs also from the donor parental strain STS **(Figure 2 A)**. However, the difference between BALB/c and CcS-5 was not significant after correction for multiple testing. Expression of *Fcgr4* in parental strains of the OcB/Dem series O20 and B10 did not differ, but the *Fcgr4* expression in the strain OcB-9 exceeded that in either parental strain, and OcB-43 expressed higher level of *Fcgr4* than the parental strain O20 **(Figure 2 A, Table 1 A).**

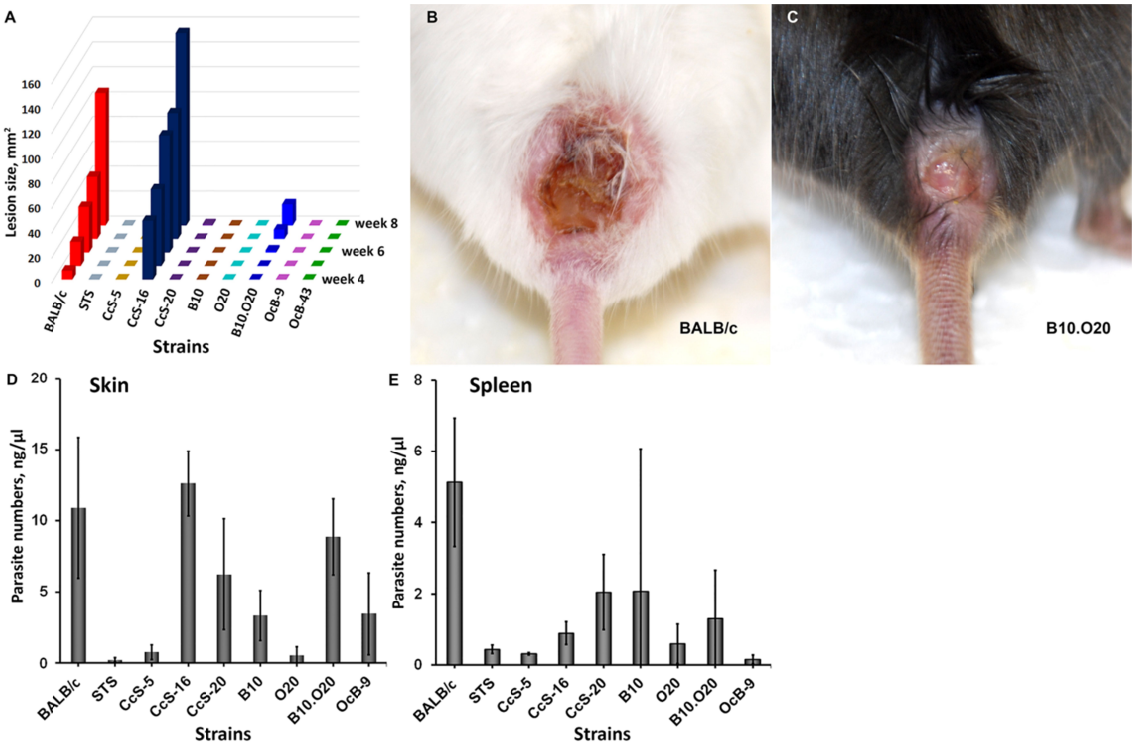

FIGURE 1. Skin lesion development and parasite load in skin and spleen of infected mice.

Kinetics of lesion development from week 4 (appearance of lesions in highly susceptible strains) till week 8 (end of experiment) (A). Median values of skin lesions of mice tested in expression experiments are shown. Skin lesion caused by *L. major* in female mouse of BALB/c strain at week 8 after infection (B). Skin lesion caused by *L. major* in female mouse of B10.O20 strain at week 8 after infection (C). Parasite load in skin (D) and spleen (E) of infected female mice of strains BALB/c (n = 5 skin, 11 spleen), STS (13 skin, 7 spleen), CcS-5 (11 skin, 4 spleen), CcS-16 (6 skin, 6 spleen), CcS-20 (7 skin, 9 spleen), O20 (6 skin, 12 spleen), B10 (9 skin, 13 spleen), B10.O20 (7 skin, 15 spleen) and OcB-9 (7 skin, 7 spleen). The data show the means ± SD. The Figure is reproduced from reference 25.

Table 1 Comparison of expression of *Fcgr4* among mouse strains of CcS/Dem and OcB/Dem series in skin

A. *Fcgr4* uninfected

CcS/Dem series

Strain	*Fcgr4* genotype	BALB/c	STS	CcS-5	CcS-16
BALB/c	C				
STS	S	0.30			
CcS-5	C	0.0082 T	0.18		
CcS-16	C	0.30	0.39	0.59	
CcS-20	C	0.0012 T	0.0022	0.065	1

OcB/Dem series

Strain	*Fcgr4* genotype	O20	B10	B10.O20	OcB-9
O20	O				
B10	B	0.37			
B10.O20	B	0.27	0.25		
OcB-9	O	0.0012 T	0.0022	0.25	
OcB-43	O	0.0012 T	0.065	0.66	0.39

B. *Fcgr4* infected

CcS/Dem series

Strain	*Fcgr4* genotype	BALB/c	STS	CcS-5	CcS-16
BALB/c	C				
STS	S	0.61			
CcS-5	C	0.69	0.95		
CcS-16	C	0.026 T	0.0012	0.0043 T	
CcS-20	C	0.0076 T	0.0047	0.0087 T	0.18

OcB/Dem series

Strain	*Fcgr4* genotype	O20	B10	B10.O20	OcB-9
O20	O				
B10	B	0.57			
B10.O20	B	0.049	0.0013 T		
OcB-9	O	0.95	0.72	0.0098	
OcB-43	O	0.97	0.61	0.014	0.90

A. Differences of *Fcgr4* expression in uninfected skin; B. Differences of *Fcgr4* expression in skin after 8 weeks of infection. Green: nominal (uncorrected) *P* value < 0.05; red: difference significant after correction for multiple testing at $P < 0.05$; T: trans-regulation of *Fcgr4* expression; C – genotype of BALB/c origin, S – genotype of STS origin, O – genotype of O20 origin, B – genotype of B10 origin.

Lymph nodes: None of the strains of the CcS/Dem series differed in expression of *Fcgr4* in lymph nodes. Expression in the strain B10.O20 was higher than in the parental strain O20 and both OcB-9 and OcB-43 **(Figure 2 B, Table 2 A)**. Strains OcB-9 and OcB-43, which did not differ in *Fcgr4* gene differed in expression of this gene **(Table 2 A)**.

Table 2 Comparison of expression of *Fcgr4* among mouse strains of CcS/Dem and OcB/Dem series in lymph nodes

A. *Fcgr4* uninfected

CcS/Dem series

Strain	*Fcgr4* genotype	BALB/c	STS	CcS-5	CcS-16
BALB/c	C				
STS	S	0.96			
CcS-5	C	1	1		
CcS-16	C	0.69	0.59	0.82	
CcS-20	C	0.86	0.94	0.82	0.82

OcB/Dem series

Strain	*Fcgr4* genotype	O20	B10	B10.O20	OcB-9
O20	O				
B10	B	0.18			
B10.O20	B	0.0047	0.073		
OcB-9	O	0.24	0.073	0.0012	
OcB-43	O	0.39	0.18	0.0012	0.015 T

B. *Fcgr4* infected

CcS/Dem series

Strain	*Fcgr4* genotype	BALB/c	STS	CcS-5	CcS-16
BALB/c	C				
STS	S	0.96			
CcS-5	C	0.098	0.13		
CcS-16	C	0.0011 T	0.24	0.0087 T	
CcS-20	C	0.0049 T	0.24	0.015 T	0.94

OcB/Dem series

Strain	*Fcgr4* genotype	O20	B10	B10.O20	OcB-9
O20	O				
B10	B	0.11			
B10.O20	B	0.000024	0.000024 T		
OcB-9	O	0.84	0.12	0.00013	
OcB-43	O	0.0010 T	0.00047	0.019	0.0025 T

A. Differences of *Fcgr4* expression in uninfected lymph nodes; B. Differences of *Fcgr4* expression in lymph nodes after 8 weeks of infection. Green: nominal (uncorrected) *P* value < 0.05; red: difference significant after correction for multiple testing at *P* < 0.05; T: trans-regulation of *Fcgr4* expression; C – genotype of BALB/c origin, S – genotype of STS origin, O – genotype of O20 origin, B – genotype of B10 origin.

Spleen: We have observed multiple differences in expression of *Fcgr4* among mouse strains in spleen **(Figure 2 C**, **Table 3 A**). BALB/c differed from STS and all tested CcS/Dem strains, exhibiting higher expression than STS, CcS-16 and CcS-20, and lower expression than CcS-5 **(Figure 2 C**, **Table 3 A)**, but the difference between BALB/c and CcS-20 was not significant after correction for multiple testing. Uninfected CcS-5 mice exceeded the expression of both parental strains and also exhibited higher expression than CcS-16 and CcS-20. OcB/Dem series: OcB-9 shown lower expression than the parental strain B10 **(Figure 2 C**, **Table 3 A)**.

Table 3 Comparison of expression of *Fcgr4* among mouse strains of CcS/Dem and OcB/Dem series in spleen

A. *Fcgr4* uninfected

CcS/Dem series

Strain	*Fcgr4* genotype	BALB/c	STS	CcS-5	CcS-16
BALB/c	C				
STS	S	0.00050			
CcS-5	C	0.00016 T	0.00067		
CcS-16	C	0.0031 T	0.18	0.0022 T	
CcS-20	C	0.035 T	0.072	0.0022 T	0.45

OcB/Dem series

Strain	*Fcgr4* genotype	O20	B10	B10.O20	OcB-9
O20	O				
B10	B	0.18			
B10.O20	B	0.38	0.093		
OcB-9	O	0.96	0.026	0.39	
OcB-43	O	0.61	0.24	0.39	1

B. *Fcgr4* infected

CcS/Dem series

Strain	*Fcgr4* genotype	BALB/c	STS	CcS-5	CcS-16
BALB/c	C				
STS	S	0.000077			
CcS-5	C	0.55	0.00011		
CcS-16	C	0.24	0.00043		
CcS-20	C	0.16	0.00032	0.0043 T	0.43

OcB/Dem series

Strain	*Fcgr4* genotype	O20	B10	B10.O20	OcB-9
O20	O				
B10	B	0.21			
B10.O20	B	0.15	0.56		
OcB-9	O	0.088	0.56		
OcB-43	O	0.78	0.016	0.0043	0.0043 T

A. Differences of *Fcgr4* expression in uninfected spleen; B. Differences of *Fcgr4* expression in spleen after 8 weeks of infection. Green: nominal (uncorrected) *P* value < 0.05; red: difference significant after correction for multiple testing at *P* < 0.05; T: trans-regulation of *Fcgr4* expression; C – genotype of BALB/c origin, S – genotype of STS origin, O – genotype of O20 origin, B – genotype of B10 origin.

Liver: Only few differences in *Fcgr4* expression were observed among strains in liver. CcS-16 exhibited higher expression than STS and CcS-5, no differences in expression were observed in OcB/Dem mice **(Figure 2 D, Table 4 A)**.

Table 4 Comparison of expression of *Fcgr4* among mouse strains of CcS/Dem and OcB/Dem series in liver

A. *Fcgr4* uninfected

CcS/Dem series

Strain	*Fcgr4* genotype	BALB/c	STS	CcS-5	CcS-16
BALB/c	C				
STS	S	0.86			
CcS-5	C	0.39	0.59		
CcS-16	C	0.15	0.015	0.0022 T	
CcS-20	C	0.22	0.24	0.39	1

OcB/Dem series

Strain	*Fcgr4* genotype	O20	B10	B10.O20	OcB-9
O20	O				
B10	B	0.065			
B10.O20	B	0.28	0.18		
OcB-9	O	0.18	0.33	0.62	
OcB-43	O	0.63	0.10	0.15	0.073

B. *Fcgr4* infected

CcS/Dem series

Strain	*Fcgr4* genotype	BALB/c	STS	CcS-5	CcS-16
BALB/c	C				
STS	S	0.000074			
CcS-5	C	0.022	0.0022		
CcS-16	C	0.000074 T	0.0022	0.0022 T	
CcS-20	C	0.58	0.065	0.70	0.065

OcB/Dem series

Strain	*Fcgr4* genotype	O20	B10	B10.O20	OcB-9
O20	O				
B10	B	0.012			
B10.O20	B	0.0036	0.19		
OcB-9	O	0.0012 T	0.0072	0.15	
OcB-43	O	0.23	0.042	0.010	0.0012 T

A. Differences of *Fcgr4* expression in uninfected liver; B. Differences of *Fcgr4* expression in liver after 8 weeks of infection. Green: nominal (uncorrected) *P* value < 0.05; red: difference significant after correction for multiple testing at *P* < 0.05; T: trans-regulation of *Fcgr4* expression; C – genotype of BALB/c origin, S – genotype of STS origin, O – genotype of O20 origin, B – genotype of B10 origin.

Skin: Infection resulted in increase of the expression of *Fcgr4* in skin of all tested strains except OcB-9 **(Figure 3 A)**. Levels of *Fcgr4* mRNA in CcS-16 and CcS-20 exceeded those in both parental strains BALB/c and STS, and also in the recombinant congenic strain CcS-5 **(Table 1 B)**, but only the differences between STS, and CcS-16 and CcS-20, and between CcS-5 and CcS-16 were significant after correction for multiple testing. Infected B10.O20 mice exhibited the highest expression from OcB/Dem series, which exceeded levels of parental strains B10 and O20, as well as those of recombinant congenic strains OcB-9 and OcB-43 **(Table 1 B)**, only difference between B10 and B10.O20 being significant after correction.

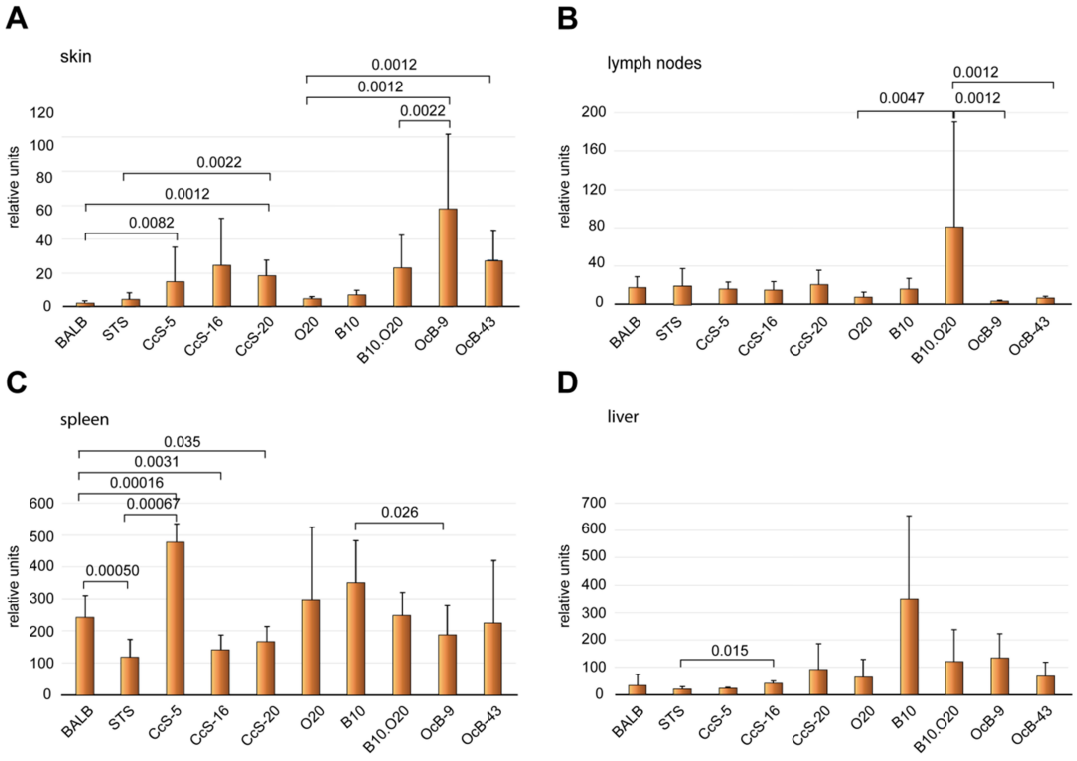

FIGURE 2. Differences in expression of *Fcgr4* in organs of uninfected mice.

Expression of *Fcgr4* in skin (A), lymph nodes (B), spleen (C) and liver (D) of uninfected female mice of strains BALB/c (n = 7 skin, 9 lymph nodes, 11 spleen, 9 liver), STS (6 skin, 9 lymph nodes, 8 spleen, 6 liver), CcS-5 (6 skin, 6 lymph nodes, 6 spleen, 6 liver), CcS-16 (6 skin, 6 lymph nodes, 6 spleen, 6 liver), CcS-20 (6 skin, 6 lymph nodes, 7 spleen, 6 liver), O20 (7 skin, 6 lymph nodes, 9 spleen, 6 liver), B10 (6 skin, 7 lymph nodes, 6 spleen, 6 liver), B10.O20 (5 skin, 7 lymph nodes, 6 spleen, 8 liver), OcB-9 (6 skin, 6 lymph nodes, 6 spleen, 5 liver) and OcB-43 (6 skin, 6 lymph nodes, 6 spleen, 7 liver) were compared. The data show the means ± SD. Only the differences between parental strains BALB/c and STS and strains of CcS/Dem series and parental strains O20, B10 and B10.O20 and strains of OcB/Dem series are shown. Nominal *P* values are shown.

Expression of *Fcgr4* mRNAs is up-regulated after infection

Lymph nodes: Up-regulation of *Fcgr4* in lymph nodes from infected mice was observed in all tested CcS/Dem strains but not in STS **(Figure 3 B)**. Strains CcS-16 and CcS-20 shown the lowest levels of *Fcgr4* and differed from both BALB/c and CcS-5 **(Figure 3 B, Table 2 B)**. However the difference from CcS-5 was not significant after correction for multiple testing. In the lymph nodes of OcB/Dem series induced *L. major* infection increased expression of *Fcgr4* in the strains B10.O20 and OcB-43 **(Figure 3 B)**. Levels of in these strains was higher than in the parental strains B10 and O20, and also than in OcB-9 **(Figure 3 B, Table 2 B)**.

Spleen: Infection also induced increase of *Fcgr4* in spleens of all CcS/Dem strains, the lowest increase being observed in the donor parental strain STS, which differed from all tested CcS/Dem strains **(Figure 3 C, Table 3 B)**. We have also observed differences between the two strains CcS-5 and CcS-20 that carry the same BALB/c-derived *Fcgr4* gene **(Table 3 B)**. Only B10.O20 exhibited increased expression of *Fcgr4* in spleens of infected strains of OcB/Dem series. The lowest level of *Fcgr4* mRNA was found in the strain OcB-43, which differs from strains B10.O20 and OcB-9; differences between OcB-43 and B10 was not significant after correction for multiple testing **(Figure 3 C, Table 3 B)**.

Liver: In liver, infection induced significant increase of *Fcgr4* mRNA in the strains of CcS/Dem series BALB/c, STS, CcS-5 and CcS-16 **(Figure 3 D**). Level of *Fcgr4* mRNA in CcS-16 is highest from all tested strains and differs from all CcS/Dem strains except CcS-20. The only observed change of expression in livers of infected OcB/Dem mice was the decrease in OcB-43. This strain differs in expression from OcB-9, which shares O20-derived *Fcgr4* gene We have also observed significant differences in expression between O20 and OcB-9 (O20 *Fcgr4* allele) and B10.O20 (B10 allele)**(Figure 3 D, Table 4 B)**.

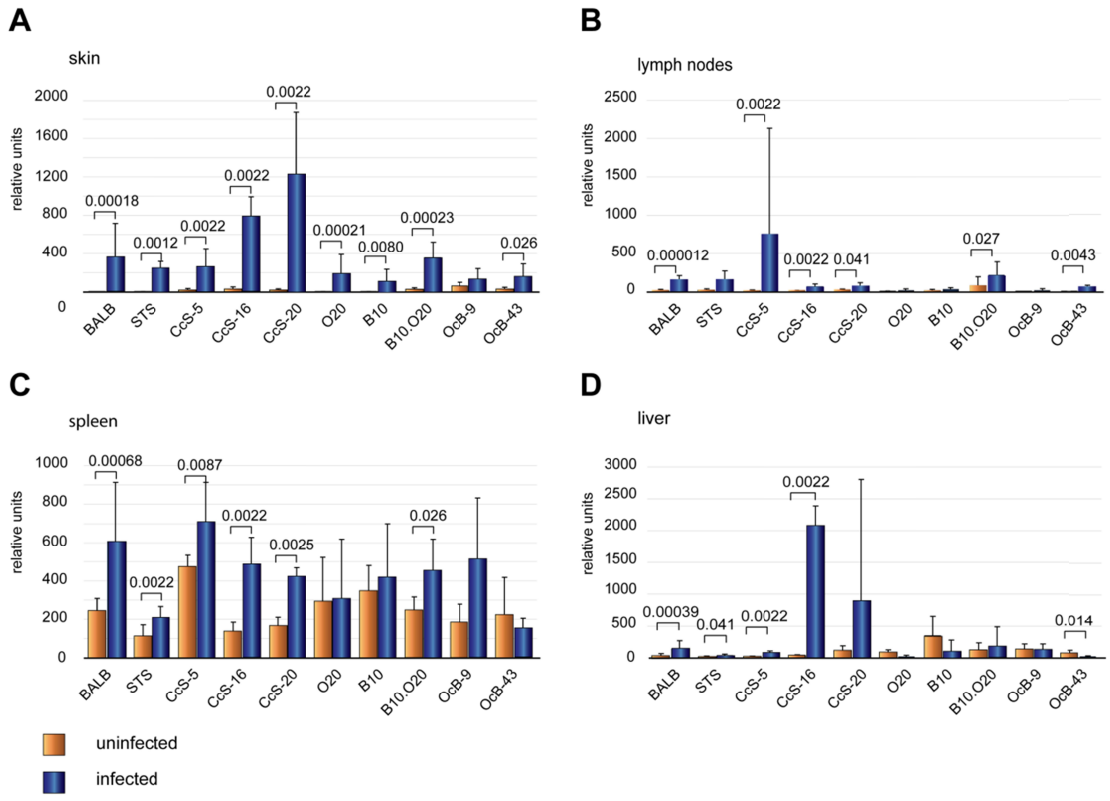

FIGURE 3. Differences in expression of *Fcgr4* in organs of uninfected and infected mice.

Expression of *Fcgr4* in skin (A), lymph nodes (B), spleen (C) and liver (D) of 8 weeks uninfected and infected female mice were compared. Numbers of tested samples from uninfected mice are given in Legend to Figure 1. Samples from infected mice used for the analysis comprised BALB/c (n = 9 skin, 11 lymph nodes, 14 spleen, 13 liver), STS (7 skin, 6 lymph nodes, 12 spleen, 6 liver), CcS-5 (6 skin, 6 lymph nodes, 6 spleen, 6 liver), CcS-16 (6 skin, 6 lymph nodes, 6 spleen, 6 liver), CcS-20 (6 skin, 6 lymph nodes, 5 spleen, 7 liver), O20 (10 skin, 9 lymph nodes, 9 spleen, 8 liver), B10 (8 skin, 13 lymph nodes, 10 spleen, 12 liver), B10.O20 (13 skin, 11 lymph nodes, 6 spleen, 11 liver), OcB-9 (7 skin, 7 lymph nodes, 6 spleen, 7 liver) and OcB-43 (9 skin, 5 lymph nodes, 6 spleen, 6 liver). The data show the means ± SD. Animals were subcutaneously inoculated with 10^7 promastigotes of *L. major.* Control, uninfected mice were kept in the same animal facility. Both

groups were killed in the same time - after 8 weeks of infection or start of experiment. The data show the means ± SD. Nominal *P* values are shown.

Immunohistochemistry analysis confirmed up-regulation of FCGR4 is livers of *L. major* infected mice

As the next step we wanted to establish whether the *Fcgr4* mRNA is translated into protein. We were also intrigued what is the relationship between FCGR4 and *L. major* parasites. To find the answer, we have compared localization of the parasites and FCGR4, as well as activated macrophages **(Figure 4)** and Ly6G+ granulocytes **(Figure 5)** in livers of uninfected and infected BALB/c and STS. Uninfected livers of both BALB/c (A) and STS (B) contained relatively low levels of FCGR4 and activated macrophages and a few Ly6G+ cells **(Figure 4 A, B; Figure 5 A, B)**. FCGR4 was present not only on activated macrophages, but also on other cell subpopulations **(Figure 4 A, B**), relatively few FCGR4 molecules were present on Ly6G+ cells **(Figure 5 A, B)**. BALB/c mice contain higher numbers of *L. major* in liver than STS mice. This infection induced higher levels of FCGR4 (Supplementary Figure 1), which partly co-localized with activated macrophages and *Leishmania* parasites **(Figure 4 C)**.

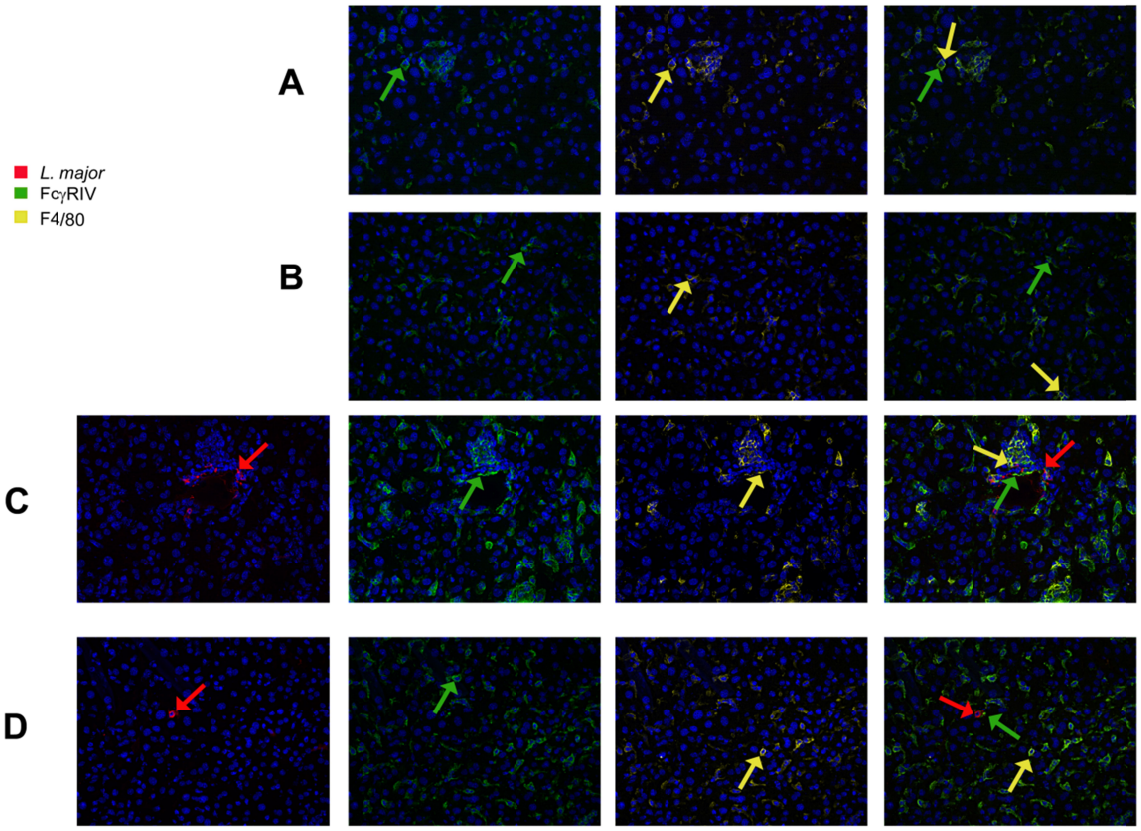

FIGURE 4. Fcγ receptor IV protein, L. major parasites and F4/80⁺ macrophages in mouse livers.

Slices of liver tissues of females of uninfected BALB/c (A), STS (B) and 8 weeks *L. major* infected BALB/c (C) and STS (D) mice were stained with the anti-*Leishmania* lipophosphoglycan mouse monoclonal antibody, the rabbit anti- CD16-2/FcγRIV monoclonal antibody and rat F4/80 monoclonal antibody as described in Materials and Methods. Nuclei of the cells were stained with bisBenzimide H33258. We evaluated 5 -10 fields from 3 infected and 3 uninfected mice of the strains BALB/c and STS used Z stack to acquire the slice in depth and to achieve plasticity. After deconvolution, we processed the image in the software ImageJ. Representative pictures for each mouse strain are presented. Red arrow points to *L. major* amastigotes (red color), green arrows show FcγRIV protein (green color), whereas yellow arrows show F4/80 (yellow color). Cell nuclei are stained in blue. Last picture in row show the merge of both channels.

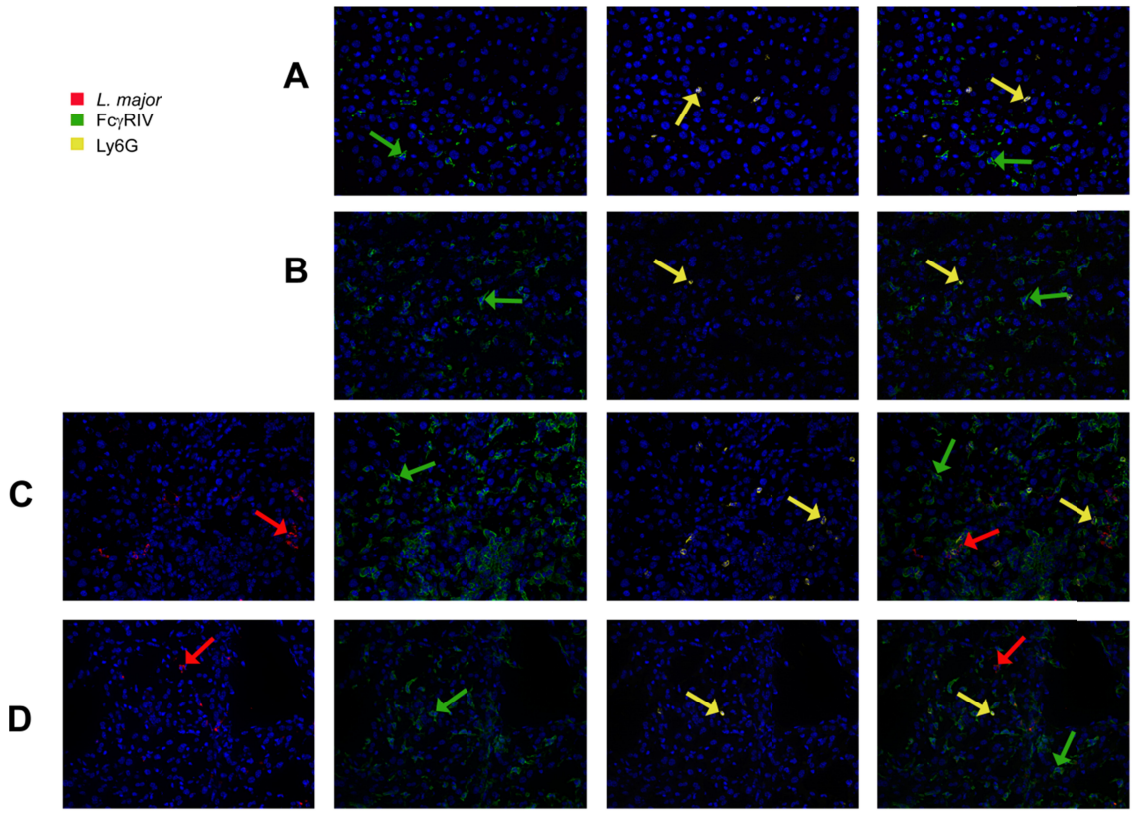

FIGURE 5. Fcγ receptor IV protein, *L. major* parasites and Ly6G+ cells in mouse livers. Slices of liver tissues of females of uninfected BALB/c (A), STS (B) and 8 weeks *L. major* infected BALB/c (C) and STS (D) mice were stained with the anti-*Leishmania* lipophosphoglycan mouse monoclonal antibody, the rabbit anti-CD16-2/FcγRIV monoclonal antibody and Ly6G(Gr1) monoclonal antibody as described in Materials and Methods. Nuclei of the cells were stained with bisBenzimide H33258. We evaluated 5 -10 fields from 3 infected and 3 uninfected mice of the strains BALB/c and STS used Z stack to acquire the slice in depth and to achieve a plasticity. After deconvolution, we processed the image in the software ImageJ. Representative pictures for each mouse strain are presented. Red arrow points to *L. major* amastigotes (red color), green arrows show FcγRIV protein (green color), whereas yellow arrows show Ly6G (yellow color). Cell nuclei are stained in blue. Last picture in row shows the merge of all channels.

Partial co-localization of *L. major* parasites and FCGR4 protein in skin

To extend these studies, we analyzed expression of FCGR4 of the strains BALB/c, STS, CcS-5, CcS-20, O20 and B10.O20 in skin of uninfected (**Figure 6**) and infected (**Figure 7**) mice. Skin of uninfected mice contained relatively low level of FCGR4. After infection, amount of FCGR4 molecules increased and part of them has been observed at the same location as *L. major* parasites (**Figure 7**). Tendency to co-localization was strongest in the strain STS (**Figure 7 B**). Part of the parasites has been in all tested strains located in activated F4/80+ macrophages. Similarly as in the liver, skin, there have been also parasites not co-localizing with FCGR4, as well as activated F4/80+ macrophages that did not harbor parasites and/or did not express FCGR4.

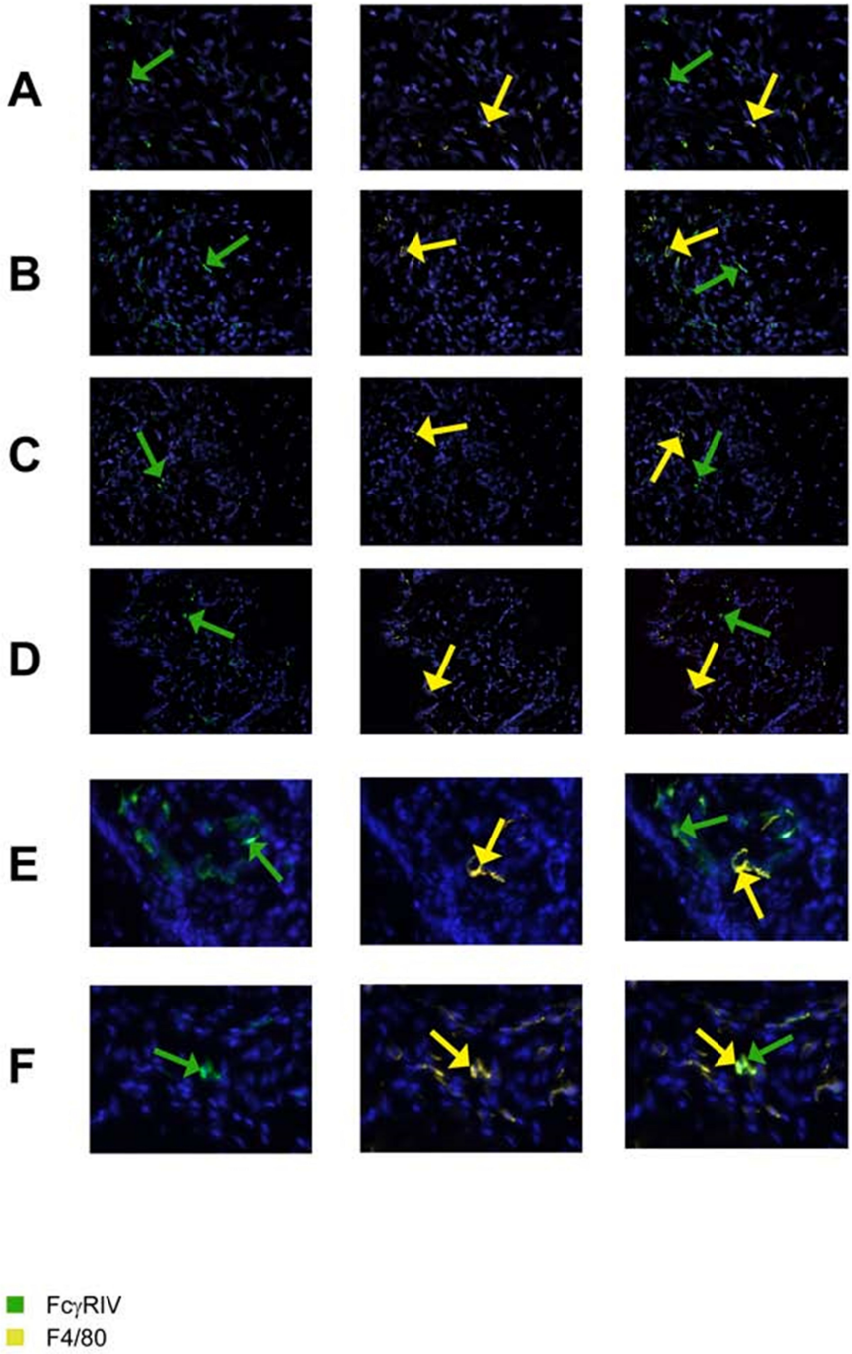

FIGURE 6. Fcγ receptor IV protein, *L. major* parasites and F4/80⁺ macrophages in mouse skin. Slices of tissues of females of uninfected BALB/c (A), STS (B), CcS-5 (C), CcS-20 (D), O20 (E), B10.O20 (F) with the rabbit anti- CD16-2/FcγRIV monoclonal antibody and rat F4/80 monoclonal antibody as described in Materials and Methods. Nuclei of the cells were stained with bisBenzimide H33258. We evaluated 5 -10 fields from 3 infected and 3 uninfected mice of each strain using Z

stack to acquire the slice in depth and to achieve plasticity. After deconvolution, we processed the image in the software ImageJ. Representative pictures for each mouse strain are presented. Green arrows show FcγRIV protein (green color), whereas yellow arrows show F4/80 (yellow color). Cell nuclei are stained in blue. Last picture in row show the merge of both channels.

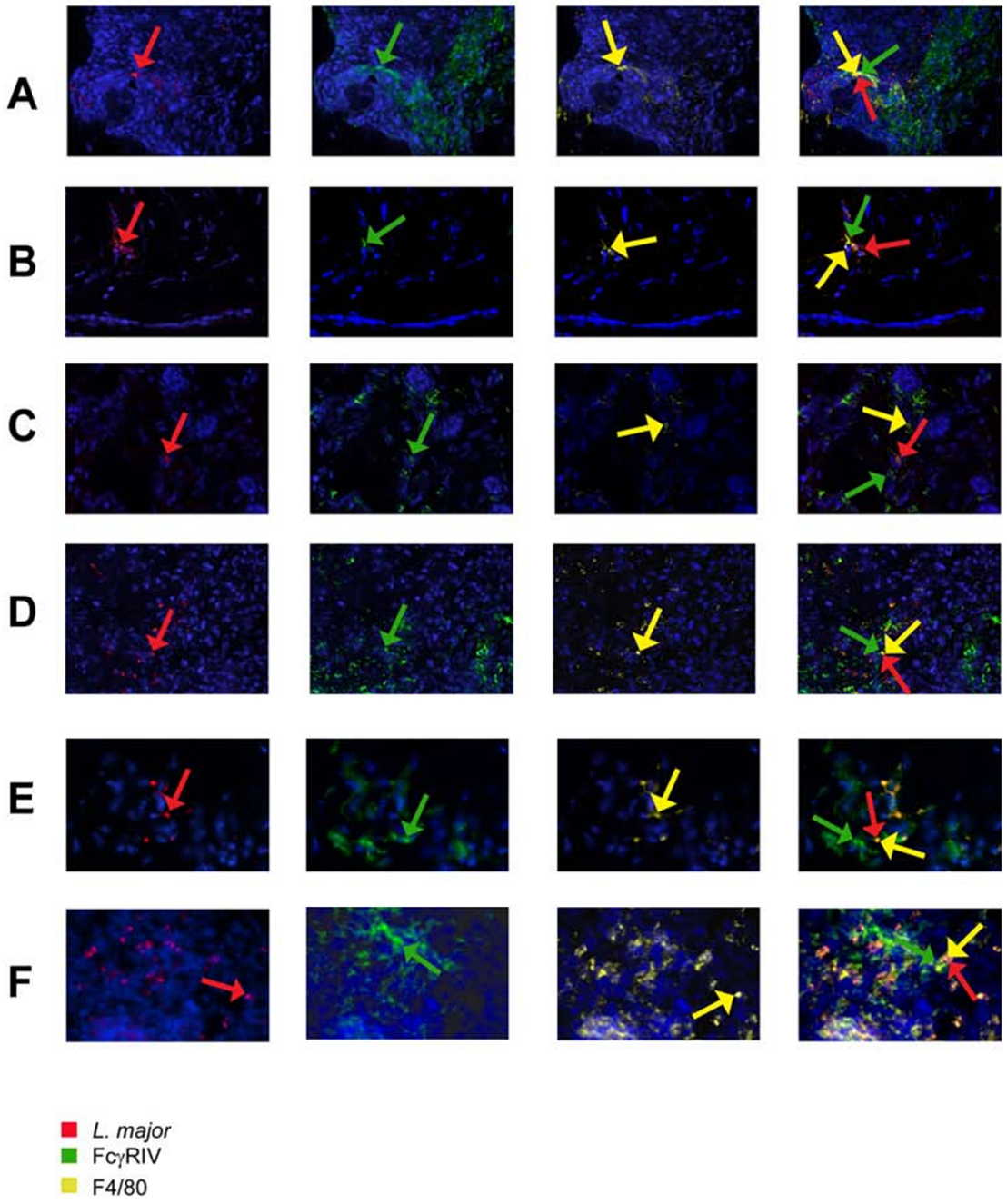

FIGURE 7. Fcγ receptor IV protein, *L. major* parasites and F4/80+ macrophages in mouse skin. Slices of tissues of females of 8 weeks *L. major* infected BALB/c (A) and STS (B), CcS-5 (C), CcS-20 (D), O20 (E), B10.O20 (F) mice were stained with the anti-*Leishmania* lipophosphoglycan mouse monoclonal antibody, the rabbit anti- CD16-2/FcγRIV monoclonal antibody and rat F4/80 monoclonal antibody as described in Materials and Methods. Nuclei of the cells were stained with

bisBenzimide H33258. We evaluated 5 -10 fields from 3 infected and 3uninfected mice of each strain and used Z stack to acquire the slice in depth and to achieve plasticity. After deconvolution, we processed the image in the software ImageJ. Representative pictures for each mouse strain are presented. Red arrow points to *L. major* amastigotes (red color), green arrows show FcγRIV protein (green color), whereas yellow arrows show F4/80 (yellow color). Cell nuclei are stained in blue. Last picture in row show the merge of all channels.

DISCUSSION

The finding that expression of *Fcgr4* increases after *L. major* infection suggests its relationship to the response to *L. major* infection. Moreover, we show partial co-localization of *L. major* parasites and FCGR4 both in liver (Figures 4 and 5) and skin (Figure 7).

In several strains the level and/or rate of increase has been linked with the *Fcgr4* allele, in others was trans-regulated, and possibly influenced by a preceding step of the response to the parasite. In this respect, *Fcgr4* appeared to resemble most other components of the response to *L. major* infection, like cytokines and antibodies, whose levels are altered in a genetically complex process (24). The dynamics of this network-like process determines the role of its individual components and hence the degree of their correlation with the outcome of the disease.

Strong genetic influence on expression of *Fcgr4*

We observed a strong genetic influence on expression of *Fcgr4*, which was revealed by genetic differences between strains. These differences were in several instances non-additive, because several CcS/Dem and OcB/Dem recombinant congenic strains exhibited levels of *Fcgr4* RNA beyond the values of their respective parental strains, i.e. either higher than the parental strain with higher expression, or lower than the parental strain with lower expression. Similar instances of a progeny strain with a quantitative phenotype, which is beyond the range of the this phenotype in its parental strains are not rare. For example, gene expression in livers of chromosome substitution mouse strains showed that only 438 out of 4209 expressed genes were within the parental strains'

range (32). This type of non-additive influence is being explained by epistastic interactions of multiple genes.

Comparison of genotypes in mouse *Fcgr* cluster on the distal mouse chromosome 1, that encodes also FCGR4 protein revealed that CcS-20 carries the same *Fcgr4* allele as BALB/c, and OcB-9 and OcB-43 the same *Fcgr4* allele as O20 **(Figure 2, Table 1A)**. However, in skin of uninfected CcS-20 mice *Fcgr4* mRNA expression differs from BALB/c, and in OcB-9 mice it differs from O20. Such differences are believed to be due to trans-regulation by distant genes (32). Thus, the presence of this *Fcgr4* allele on the chromosome 1 in strain CcS-20, which differs from BALB/c at several parts of the genome, where it carries STS-derived genes, suggests that the *Fcgr4* expression different from the parental strain BALB/c reflects regulation by these STS derived non-*Fcgr4* genes (*trans*-regulation). Similarly, OcB-9 has *Fcgr4* allele of O20 (O), but the two strains differ in *Fcgr4* expression, which indicated a likely trans-regulation of expression by B10-derived genes outside *Fcgr* cluster **(Figure 2 A, Table 1 A**). In uninfected mice we observed in total 11 instances of *trans*-regulation **(Tables 1A-4A).**

L. major infection can activate new regulatory processes, which can partly mask these original trans-regulatory processes present in uninfected mice. This was indicated by a new pattern of strain differences that revealed 6 novel instances of *trans*-regulation **(Tables 1B-4B)**. Additional genetic analyses are needed to elucidate these changes of regulation after infection.

Important role of genetic background in regulation of FcγR function was indicated also by a different outcome of experiments with mice with deleted common FcγR chain, leading either to development of protective immunity or to aggravation of disease manifestations depending of genotype of mammalian host (18, 19).

Mouse strains vary in their pattern of response to *L. major* infection (24) and accordingly in expression of a large number of molecules. Such phenotypic variability is mostly based on genetic

polymorphism (33). RC strains used in the current experiments were shown previously to differ in many phenotypic traits including response of splenocytes to IL-2 and anti-CD3 (34), and in mixed lymphocyte cultures (35, 36).

Organ-specific response

We have observed different *Fcgr4* responses among tested strains in the four tested organs (Figures 2 and 3). This is not unique. Organ-specific responses in susceptibility to *L. major* were described both in genetic (28, 37, 38, 39) and mechanistic (40, 41) studies. These differences in responses might be caused by different cellular organ composition and/or microenvironment and might be effectuated by mediators produced by differentially activated cells that influence defense pathways.

Genetic evidence of FCGR4 role in inflammatory response in leishmaniasis

Leishmania parasites are found in organs of infected susceptible mice with manifest disease but also in resistant mouse strains without clinical symptoms (15, 25, 26, 40, 42). It is not yet known whether the changes in *Fcgr4* expression after *L. major* infection and their modifying effect on its outcome reflect a direct inhibitory interaction of this molecule with the parasite, or whether they operate through indirect modification of the immune responsiveness. Persistent parasites can contribute to the maintenance of protective immunity (15), but their presence might also stimulate chronic inflammatory response and/or present danger to the host (15). It is therefore important to determine, whether the people carrying persistent *Leishmania* parasites have an increased susceptibility other immune-related diseases. FCGR4 is involved also in autoimmune diseases - autoimmune tissue injury (43), acute glomerular inflammation (44), autoantibody-induced arthritis (45), whereas its human ortholog FCγRIIIA (CD16A) is involved in cancer susceptibility (46,47). The immune reactions associated with persistent *Leishmania* infection may have considerable importance, because besides the 12 million people in the world who have clinically manifest leishmaniasis (14), there are estimated 120 million infected people with no disease symptoms (15).

Conclusions

We present the first data on expression of *Fcgr4* during leishmaniasis. They revealed a strong genetic control of *Fcgr4* expression in uninfected as well as *L. major* infected mice. In certain strains it involved also trans-regulation by non-*Fcgr4* genes. In several organs, expression of *Fcgr4* in recombinant congenic strains were outside the range of the parental strains. Interestingly, the *Fcgr4* cluster is linked to the *Lmr20* locus on chromosome 1 that significantly influences the susceptibility to *L. major* infection (38). Further genetic and functional analysis such as elimination of specific antigen-bearing cell subpopulation (48), specific *in vivo* knockdown of proteins by intrabodies (49), using transcriptional gene modulators (50), functional inactivation (51), iRNA (52) and by CRISPR-mediated control of gene repression and activation (53) may help to identify and manipulate these as yet unknown genes.

AUTHORS' CONTRIBUTIONS

HH designed and performed immunohistochemistry analysis and analyzed the data, YS planned and performed parasitology and expression experiments and contributed to the writing of the manuscript, VV performed parasitology experiments and analyzed the data, IK contributed to genetic analysis of tested strains, MS performed parasitology experiments, PD analyzed the data and contributed to the writing of the manuscript, ML conceived the study, interpreted data and wrote the manuscript. All authors reviewed the manuscript.

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Spotlight

Mannose Receptor and the Mystery of Nonhealing *Leishmania major* Infection

Yahya Sohrabi[1,2,*] and Marie Lipoldová[2,*]

Scientists have long puzzled over the ability of *Leishmania major* Seidman (LmSd) to form nonhealing cutaneous lesions in the face of a strong Th1 response. A recent study identified a population of dermal macrophages that are preferentially infected by LmSd in a mannose receptor 1-, C-type 1 (MRC1/CD206)-dependent manner.

Control and prevention of leishmaniasis is a major challenge in more than 98 countries on five continents. Environmental factors, mammalian host genetic background, and the species and/or strain characteristics of the parasite make the host–parasite interaction very complex. In general, cutaneous leishmaniasis is characterized by a self-healing lesion, which heals over a period of time [1]. However, in rare cases, infection can result in a nonhealing form of the disease that can persist for several years and does not respond to any of the existing chemotherapies [2]. One of the strains that cause this serious form of the disease – *Leishmania major* Seidman (MHOM/SN/74/SD) (LmSd) – was isolated 45 years ago from a Peace Corps volunteer in Senegal. The patient exhibited chronic cutaneous lesions with persisting parasites despite a strong cell-mediated immune response to *Leishmania* antigens [3]. This enigmatic response was reproduced in mice; the mouse strain C57BL/6, which is typically resistant to *L. major*, developed nonhealing cutaneous lesions after infection with LmSd, while exhibiting a strong Th1 response that was characterized by high levels of IFN-γ and an absence of IL-4 and IL-13 [4]. Interestingly, it was shown that the Nlrp3 inflammasome, IL-1β, and neutrophil recruitment were required for susceptibility to LmSd [5], but the trigger for these processes was unclear. The answer has now been provided in a new study by Lee and colleagues [6]. These authors identified a population of $MRC1^{hi}$ dermal macrophages that were preferentially infected by LmSd. Infection was dependent on the mannose receptor 1, C-type 1 (MRC1/CD206), and promoted a nonhealing cutaneous disease.

MRC1 is an endocytic receptor that recognizes sulfated and mannosylated sugars and is involved in neutralization of pathogens, antigen presentation, cell–cell recognition, serum glycoprotein turnover, and other biological processes. It is expressed mainly by subpopulations of macrophages and dendritic cells [7].

The authors [6] first compared uptake and replication of LmSd and *L. major* Friedlin (MHOM/IL/80/Friedlin) (LmFn), which cause nonhealing and healing lesions in C57BL/6 mice, respectively, using M-CSF-induced bone-marrow-derived macrophages (BMDMs). The uptake of LmSd was higher than the uptake of LmFn, whereas replication of both *L. major* strains was similar. Various receptors, including MRC1, fibronectin receptor, or complement receptor 3, can mediate phagocytosis of parasites by macrophages, therefore the individual receptors were blocked before infection. There was no difference between uptake via fibronectin or complement receptor 3 of either strain, whereas blocking of MRC1 led to decreased uptake of LmSd. The role of this receptor in uptake of LmSd was confirmed by using BMDMs from MRC1-deficient mice (*Mrc1*−/−), which exhibited dramatically low parasite uptake of LmSd but no change in LmFn. MRC1 was previously described as a marker of M2 macrophages, a subgroup of macrophages with anti-inflammatory phenotypes; by contrast, macrophages with proinflammatory effects are often referred to as M1. It is important to notice that the M1 versus M2 paradigm is utilized in mice and could be translated to some degree to humans [8]. The authors treated BMDMs with M1 stimuli [lipopolysaccharide (LPS) or IFN-γ plus LPS]. This led to the decrease of MRC1 expression and a loss of difference between LmSd and LmFn uptake. On the other hand, treatment of BMDMs with IL-10, or IL-4 and IL-10, enhanced MRC1 expression and favored uptake of LmSd. In addition, these cytokines are prerequired for the local maintenance of a P4 macrophage population during infection.

The next step was characterization of MRC^{hi} macrophages from the dermis. These cells were identified as P4 dermal macrophages, which are defined by their profile of cell-surface markers as $CD11b^{+}MRC^{hi}Ly6C^{int}CD64^{+}CCR2^{low}M\text{-}HCII^{-}$. They showed characteristics of mature macrophages with abundant cytoplasmic vacuoles and melanin granules, and they exhibited high phagocytic activity. P4 was the predominant subset infected by LmSd after infection in the mouse ear. Analysis of the ontogeny of this P4

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SYMPOSIUM-IN-WRITING PAPER

MDSCs in infectious diseases: regulation, roles, and readjustment

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Abstract

Many pathogens, ranging from viruses to multicellular parasites, promote expansion of MDSCs, which are myeloid cells that exhibit immunosuppressive features. The roles of MDSCs in infection depend on the class and virulence mechanisms of the pathogen, the stage of the disease, and the pathology associated with the infection. This work compiles evidence supported by functional assays on the roles of different subsets of MDSCs in acute and chronic infections, including pathogen-associated malignancies, and discusses strategies to modulate MDSC dynamics to benefit the host.

Keywords Myeloid regulatory cells · MDSC · Infection · Immunosuppression · Oncogenic viruses · Mye-EUNITER

Abbreviations

Arg	Arginase
Arm	Armstrong
ATRA	All-trans retinoic acid
B. fragilis	*Bacteroides fragilis*
C. albicans	*Candida albicans*
C13	Clone 13
CCR	C-C Chemokine receptor
COST	European Cooperation in Science and Technology
EBV	Epstein Barr virus
ETBF	Enterotoxigenic *Bacteroides fragilis*
FV	Friend virus
H. felis	*Helicobacter felis*
H. polygyrus	*Heligmosomoides polygyrus*
HbsAg	HBV surface antigen
HDT	Host-directed therapy
IAV	Influenza A virus
iNKT	Invariant NK T
JEV	Japanese encephalitis virus
K. pneumoniae	*Klebsiella pneumoniae*
L. major	*Leishmania major*
LCMV	Lymphocytic choriomeningitis virus
LOX	Lipoxygenase
Mφ	Macrophage
M-MDSC	Monocytic MDSC
M. tuberculosis	*Mycobacterium tuberculosis*
MR	Mannose receptor
MRC	Myeloid regulatory cell
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NOS	NO synthase
P. aeruginosa	*Pseudomonas aeruginosa*
PcP	*Pneumocystis* pneumonia
PDE	Phosphodiesterase
PGE2	Prostaglandin E2
PMN-MDSC	Neutrophil-like MDSC
ROS	Reactive oxygen species
S. aureus	*Staphylococcus aureus*
SIV	Simian immunodeficiency virus
T. crassiceps	*Taenia crassiceps*
T. cruzi	*Trypanosoma cruzi*
T. gondii	*Toxoplasma gondii*
TB	Tuberculosis
Tfh	T Follicular helper

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Novel Loci Controlling Parasite Load in Organs of Mice Infected With *Leishmania major*, Their Interactions and Sex Influence

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Leishmaniasis is a serious health problem in many countries, and continues expanding to new geographic areas including Europe and USA. This disease, caused by parasites of *Leishmania* spp. and transmitted by phlebotomine sand flies, causes up to 1.3 million new cases each year and despite efforts toward its functional dissection and treatment it causes 20–50 thousands deaths annually. Dependence of susceptibility to leishmaniasis on sex and host's genes was observed in humans and in mouse models. Several laboratories defined in mice a number of *Lmr* (*Leishmania major response*) genetic loci that control functional and pathological components of the response to and outcome of *L. major* infection. However, the development of its most aggressive form, visceral leishmaniasis, which is lethal if untreated, is not yet understood. Visceral leishmaniasis is caused by infection and inflammation of internal organs. Therefore, we analyzed the genetics of parasite load, spread to internal organs, and ensuing visceral pathology. Using a new PCR-based method of quantification of parasites in tissues we describe a network-like set of interacting genetic loci that control parasite load in different organs. Quantification of *Leishmania* parasites in lymph nodes, spleen and liver from infected F_2 hybrids between BALB/c and recombinant congenic strains CcS-9 and CcS-16 allowed us to map two novel parasite load controlling *Leishmania major response* loci, *Lmr24* and *Lmr27*. We also detected parasite-controlling role of the previously described loci *Lmr4*, *Lmr11*, *Lmr13*, *Lmr14*, *Lmr15,* and *Lmr25,* and describe 8 genetic interactions between them. *Lmr14*, *Lmr15*, *Lmr25,* and *Lmr27* controlled parasite load in liver and lymph nodes. In addition, *Leishmania* burden in lymph nodes but not liver was influenced by *Lmr4* and *Lmr24*. In spleen, parasite load was controlled by *Lmr11* and *Lmr13*. We detected a strong effect of sex on some of these genes. We also mapped additional genes controlling splenomegaly and hepatomegaly. This resulted in a systematized insight into genetic control of spread and load of *Leishmania* parasites and visceral pathology in the mammalian organism.

Keywords: *Leishmania major*, visceral leishmaniasis, parasite load, PCR-ELISA, susceptibility to Infection, QTL, mouse model, sex influence

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INTRODUCTION

Leishmaniasis is a neglected tropical disease, which belongs to the top health problems because it is endemic in 98 countries in Asia, Africa, the Americas, and the Mediterranean region (1–3) and is gradually expanding to new areas, including Central Europe and USA (2, 4–9). The disease occurs in cutaneous, mucocutaneous, and visceral forms (9). It is caused by the protozoan intracellular parasite *Leishmania* transmitted by *Phlebotomus* spp. in the Old World and *Lutzomyia* spp. in the New World. The parasite can infect about 70 species of vertebrates, including humans (10–13). In addition, there are specific groups of asymptomatic infection (14), and post-kala-azar dermal leishmaniasis (15). Visceral leishmaniasis is fatal in more than 95% of cases if left untreated (9). Up to 1.3 million new cases occur annually: 300 000 are visceral and 1 million are cutaneous and mucocutaneous and about 20–50 thousands patients die (13). In the infected mammalian organism, *Leishmania* parasites invade "professional phagocytes," including monocytes, macrophages, and neutrophils and can also reside in dendritic cells (DC) (16), immature myeloid precursor cells, hepatocytes, and fibroblasts; the parasite can also enter sialoadhesin-positive stromal macrophages (17).

Treatment of leishmaniasis is difficult because of the lack of reliable drugs. Existing leishmanicidal agents show severe side effects. In addition, the treatment is costly and not readily available to a majority of patients. In spite of numerous attempts to develop vaccination against leishmaniasis, there are still no safe and effective vaccines suitable for humans (18, 19). Clinical form and susceptibility to leishmaniasis are dependent on parasite species, environmental and social factors, and also on nutrition and genotype of the host (3, 10, 16, 20).

Parasite load is one of the most important parameters of leishmaniasis determining the course of infection and the degree of susceptibility. However, the information about genetic control of parasite load remains incomplete and fragmented; there is no systematic description of the control of parasite load in combination with other pathological parameters and influence of sex on these genes is not known for any of the studied *Leishmania* species. The use of mouse models in studies of selected candidate genes and also for hypothesis-generating genome-wide association and linkage analysis, revealed several genes and loci controlling parasite burden (16, 21–23) (**Table 1**). However, quantification of parasites had been a laborious task providing inaccurate results due to technical problems. These problems were reduced with development of sensitive PCR-based assays (18), which permitted to perform genome wide-search (21, 23).

TABLE 1 | Genetic control of parasite load in mouse leishmaniasis.

Parasite	Organ	Gene/Locus	References
L. donovani	Spleen	*Slc11a1* (weak effect), *H2*, *Lyst*	(24, 25)
	Liver	*Slc11a1, H2, Ir2*	(25–28)
	Bone marrow	*H2*	(25)
L. mexicana	Spleen	*Slc11a1, H2*	(29)
	Liver	*Slc11a1, H2*	(29)
L. infantum	Spleen	*Slc11a1, H2*	(25)
	Liver	*Slc11a1, H2*	(25)
	Bone marrow	*H2*	(25)
L. major	Spleen	*Lmr5*	(21)
	Skin	*Dice1.2*	(30)
	Lymph nodes	*Lmr20*	(21)
L. tropica	Spleen	*Ltr3, Ltr6*	(23)
	Liver	*Ltr2, Ltr4, Ltr8*	(23)
	Lymph nodes	*Ltr1, Ltr4*	(23)

Leishmania species and strains used in described experiments: (21), L. major (LV 561 (MHOM/IL/67/LRC-L137 JERICHO II); (23), L. tropica (MHOM/1999/TR/SU23); (24), L. donovani 2S (MHOM/SD/61/2S); (25), L. infantum, zymodem MON-1, L. donovani L82 (LV9) (MHOM/ET/67/L82); (26), L. donovani L82 (LV9) (MHOM/ET/67/L82); (27), L. donovani L82 (LV9) (MHOM/ET/67/L82); (28), L. donovani, 3S (MHOM/SD/62/3S); (29), L. mexicana (MNYC/B2/62/M379); (30) L. major (WHOM/IR/-/173).

We explored genetic control of parasite load in different organs after *L. major* infection. This parasite is the predominant causative agent of human cutaneous leishmaniasis in the Old World, in rare cases it can visceralize in an immunocompromised (HIV infected) host (31), but *L. major* strain (MRHOM/IR/75/ER) was described to visceralize also in an immunocompetent individual (32). Instances of *L. major* visceralization in non-immunocompromised people may suggest the presence of genetic factors determining extreme forms of high susceptibility to *L. major* infection. Infection by *L. major* in mouse is controlled by multiple genes. These multiple genes-loci have been mapped in three different resistant strains—C57BL/10Sn (B10.D2), C57BL/6 and STS—using the susceptible strain BALB/c for mapping in each case (16, 22); in cross with B10.D2 using for infection *L. major* (strain WHOM/IR/-/173), in crosses with C57BL/6 and STS *L. major* V121 (the cloned line V121 derived from MHOM/IL/67/Jericho II) and *L. major* strain (LV 561 (MHOM/IL/67/LRC-L137 JERICHO II), respectively. These experiments revealed 26 *Lmr* (*Leishmania major* response) and 5 *Lmrq* (*Leishmania major* resistance QTL) loci that determine skin lesion size, splenomegaly, hepatomegaly, cytokine levels in blood serum, eosinophil infiltration into lymphatic nodes, and parasite numbers in different organs (16, 21, 22, 33). Several loci (*Lmr4*/*Lmrq1*, *Lmr5*/*Lmrq3*, *Lmr6*/*Lmrq4,* and *Lmr12*/*Dice1b*) detected in crosses with resistant strains STS and B10.D2 overlap, which might indicate in these loci not only general response across different mouse strains, but also general response to different *L. major* strains.

In the present study, we tested parasite load and dissemination in F_2 hybrids of BALB/c and recombinant congenic mouse strains CcS-9 and CcS-16, infected by *L. major* LV561. The disease development during previous experiments showed no significant difference between females and males of the CcS-16 strain after

Abbreviations: BALB, (Bagg and Albino) a standard inbred mouse strain; CcS/Dem, BALB/c-c-STS/Dem (recombinant congenic strain); *Dice1.2*, determination of interleukin 4 commitment 1b; *H2*, histocompatibility-2; IL, interleukin; IFNγ, interferon gamma; IgE, immunoglobulin E; *Lmr, Leishmania major* response; *Ltr, Leishmania tropica* response; PCR-ELISA, polymerase chain reaction enzyme linked immunosorbent assay; QTL, quantitative trait locus; RC, recombinant congenic; *Slc11a1,* solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1; SNB-9, saline-neopeptone-blood-9; STS, an inbred mouse strain of Swiss origin.

infection with *L. major* (34); however the influence of sex was present in CcS-9 strain (33). These differences determined selection of sex of mice used in the present F_2 hybrid study. The current study aims to provide a first systematic a genome wide search description of the genetic control of parasite load in mammalian organs after *L. major* infection.

MATERIALS AND METHODS

Mice

Recombinant congenic (RC) strains of mice of the BALB/c-c-STS/Dem (CcS/Dem) series, containing random distinct segments of 12.5% STS/A (STS) genes on the background of BALB/cHeA (BALB/c) genome (35), exhibit various susceptibility to *Leishmania* infection and proved to be a powerful tool in research of genetic control of the disease (16, 21, 23, 33, 36, 37). BALB/c is a standard inbred mouse strain, STS is an inbred mouse strain of Swiss origin. The parts of CcS-9 and CcS-16 genomes inherited from the BALB/c or STS parents were defined (35).

At the time of experiments, mice of CcS-9 strain were in the 40 generation of inbreeding, and therefore highly homozygous. F_2 hybrids between CcS-9 and BALB/c (age 11–21 weeks at the time of infection, mean and median age 14.8 and 15 weeks, respectively) were produced at the Institute of Molecular Genetics; 254 F_2 hybrids between BALB/c and CcS-9 comprised 139 females and 115 males. Mice were tested in three independent experimental groups; male and female mice were placed into separate rooms, and males were caged individually.

In CcS-16 experiments, only females were used due to absence of sex differences in previous experiments. Mice of CcS-16 strain were in the generation 37 of inbreeding, and therefore highly homozygous. We produced 577 female F_2 hybrids between CcS-16 and BALB/c (age at the time of infection, 14–17 weeks) and tested them in four independent experiments.

Mice were kept in the animal facility of Institute of Molecular Genetics AS CR.

Parasites

Leishmania major LV 561 (MHOM/IL/67/LRC-L137 JERICHO II) was maintained in rump lesions of BALB/c females. Amastigotes were transformed to promastigotes using SNB-9 (38); 10^7 promastigotes from 6 days old subculture 2 were inoculated in 50 μl sterile saline s.c. into mouse rump (39).

Disease Phenotype

The size of the primary skin lesions was measured weekly using a Vernier caliper gauge. Mice were euthanized 8 weeks after infection and body, spleen, and liver weights were recorded. The blood, spleen, liver, and inguinal lymph nodes were collected for the further analysis. Splenomegaly (enlargement of the spleen) and hepatomegaly (enlargement of the liver) were calculated as organ-to-body weight ratio × 1000. Parasite load was presented in relative units as concentration of parasite DNA in ng per 1 μl.

Cytokine and IgE Levels

IgE, IL-4, IL-10, IL-12, IL-13, and IFNγ levels in serum were determined using the primary and secondary monoclonal antibodies (IgE: R35-72, R35-118, IL-4: 11B11, BVD6-24G2, IL-10: JES5-2A5, JES5-16E3, IL-12: C15.6, C17.8, IFNγ: R4-6A2, XMG1.2) and standards from Pharmingen (San Diego, CA, USA) (purified mIgE: C38-2, recombinant mouse IL-4, mIL-10, mIL-12 p70 heterodimer, and mIFNγ). The enzyme-linked immunosorbent assay (ELISA) was performed as recommended by Pharmingen. IL-13 level in serum were determined using the Murine IL-13 ELISA Development Kit 900-K207 (by PeproTech EC (London, United Kingdom), which contained both primary and secondary monoclonal antibodies and standard and the ELISA was performed as recommended by PeproTech EC. The IL-4, IL-10, IL-12, IL-13, IFNγ, and IgE levels were estimated using the curve fitter program KIM-E (Schoeller Pharma, Prague, Czech Republic).

Genotyping of F_2 Hybrids

The frozen archive material of F_2 crosses genotyped in previous mapping experiments (33, 40) was used for measurement of parasite DNA and analysis of genetic linkage.

In CcS-9 experiment, STS derived segments were typed in F_2 hybrids using 19 microsatellite markers on eight chromosomes: D2Mit148, D2Mit283, D4Mit7, D4Mit17, D4Mit23, D4Mit53, D4Mit172, D5Mit24, D5Mit143, D6Mit122, D6Mit274, D9Mit15, D11Mit141, D11Mit242, D11Nds10, D11Nds18, D16Mit19, D17Mit120, and D17Mit122 as described elsewhere (33).

In CcS-16 experiment, the segments of STS origin on 9 chromosomes were typed in F_2 hybrids using 23 markers: D2Mit51, D2Mit102, D2Mit156, D2Mit283, D2Mit389, D2Nds3, D3Mit11, D3Mit25, D4Mit153, D6Mit48, D6Mit320, D10Mit67, D10Mit103, D11Mit37, D11Mit139, D11Mit242, D16Mit126, D17Mit38, D17Mit130, D18Mit35, D18Mit40, D18Mit49, and D18Mit120 as described in Vladimirov et al. (40).

Measurement of Parasite Load in Organs

Total DNA was isolated from frozen lymph nodes, spleen, and liver samples, and parasite load was measured using PCR-ELISA according to the previously published protocol (41). Briefly, total DNA was isolated using a standard proteinase procedure (42). 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. In CcS-9 experiment, 26-cycle PCR reaction was used for quantification of parasites in lymph nodes and spleen, and 35 cycles for liver. In CcS-16 experiment, 24-cycle PCR reaction was used for quantification of parasites in lymph nodes; 28 cycles for spleen, and 33 cycles for liver. Parasite load was determined by measurement of the PCR product with

the modified ELISA protocol (Pharmingen, San Diego, USA). 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 (21, 41).

Statistical Analysis

The role of genetic factors in control of parasite dissemination was estimated with ANOVA using Statistical for Windows 12.0 (StatSoft, Inc., Tulsa, OK, USA). Markers and interactions with $P < 0.05$ were combined in a single comparison. Genotype (marker), sex and age were fixed factors and the experiment was random factor. The time course of skin lesion development was evaluated on the basis of weekly measurements of lesion size in each mouse in weeks 4–8 after infection. Variance components and mixed model ANOVA of Statistica for Windows 12.0 (StatSoft, Inc., Tulsa, OK, USA) with marker as the fixed variable and the week of observation as the covariate have been used to evaluate the linkage.

When necessary, the original values of an analyzed parameter were transformed for normalization of the distribution as described in the legends to the Tables. For whole genome significance values (corrected *P*-values), the observed *P*-values (αT) were adjusted using the following formula (43):

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

In the present formula, 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) or C = 9 (between CcS-16 and BALB/c); ρ = 1.5 for F_2 hybrids; h(T) = the observed statistic (F ratio).

The percent of the phenotypic variance explained by a certain locus or an interaction between loci was calculated subtracting the 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:

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

RESULTS

The present studies revealed two novel *Lmr* loci, new functions of six previously mapped *Lmr* loci and described multiple and heterogeneous genetic effects influencing parasite dissemination into the host organism. Because the STS-derived regions in CcS-9 and CcS-16 are different, we detected in their respective F_2 hybrids with BALB/c different loci controlling parasite load.

The CcS-9 Strain: Two Novel *Lmr* Loci, Multiple Interactions, and Sex Dependent Control of Parasite Load

In F_2 hybrids prepared from the parental strain BALB/c and the RC strain CcS-9, the analysis of parasite load in inguinal lymph nodes, spleen and liver followed by linkage analysis, revealed both main effect loci and interactions of genes located on chromosomes 2, 4, 5, 6, 11, and 17. Two novel *Lmr* loci, *Lmr24,* and *Lmr27* were detected in the CcS-9 experiments (**Figure 1**).

Both in males and females, a homozygous STS allele (SS) at the *Lmr14* linked to the marker D2Mit283 determined the highest parasite load in inguinal lymph nodes interacting with homozygous STS alleles (SS) of the *Lmr25* linked to D5Mit143 (corrected $P = 0.0314$, 2.1% of variance explained), and also with the *Lmr24* linked to D4Mit172 (corrected $P = 0.0247$, 2% of variance explained) (**Table 2**).

Genetic control of parasite load in lymph nodes was strongly dependent on sex (**Tables 3**, **4**): in interactions of sex with markers D11Nds10, D11Nds18, D11Mit141, D2Mit148, D17Mit122 - $P < 10^{\wedge(-30)}$; D11Nds10 and D17Mit122, as well as D6Mit122 and D17Mit122 - $P < 10^{(\wedge-30)}$. In females, the highest parasite load in lymph nodes were observed in mice homozygous for BALB/c (CC) allele in the *Lmr15* linked to D11Nds10 (corrected $P = 0.000696$, 7.42% of variance explained) (**Table 3A**). In males, a homozygous STS allele (SS) of the *Lmr14* linked to D2Mit148 (corrected $P = 0.031$, 10.5% of variance explained), and the novel locus *Lmr27* linked to D17Mit122 (corrected $P = 0.012$, 15.3% of variance explained) determined higher parasite load in inguinal lymph nodes (**Table 3B**). Homozygotes in SS allele on the novel locus *Lmr27* linked to D17Mit122 determined the highest parasite load in males in two interactions: with CC allele of the *Lmr4* linked to D6Mit122 (corrected $P = 0.00427$, 7.4% of variance explained), and CS allele on the *Lmr15* linked to D11Nds10 (corrected $P = 0.0261$, 6% of variance explained), respectively (**Table 4**).

No sex differences were detected in control of parasite load in liver. Parasite load in liver were therefore calculated for total group (both males and females), and revealed the effect of the CC allele on *Lmr15* linked to D11Mit141 (corrected $P = 0.0037$, 8.6% of variance explained) and D11Nds10 (corrected $P = 0.0011$, 9.6% of variance explained) in control of higher parasite burden (**Table 5**). Parasites load in liver was also controlled by an interaction between the novel locus *Lmr27* linked to D17Mit120 and *Lmr14* linked to D2Mit283 (corrected $P = 0.0184$, 6.9% of variance explained) (**Table 6**) and by an interaction of *Lmr27* linked to D17Mit122 with *Lmr25* linked to D5Mit143 (suggestive linkage; corrected $P = 0.0640$, 6.2% of variance explained). Lowest parasite load are linked with the interaction of homozygotes in STS (SS) allele at *Lmr25* with the homozygotes in BALB/c (CC) allele at *Lmr27*.

No loci that determine parasite load in spleen were found in F_2 hybrids derived from the CcS-9 strain.

Loci Controlling Organ Pathology and Immune Response in CcS-9

Analysis of different parameters of the disease indicated a novel *Lmr* locus which controls organ pathology and systemic immune response (**Table 7**). *Lmr24* on chromosome 4 influenced splenomegaly, levels of IFNγ, and IL-4 in serum and development of skin lesions. Control of splenomegaly was linked with the marker D4Mit23 (corrected $P = 0.0426$, 4.76% of variance explained), skin lesion size (kinetics of development of skin lesions)—with D4Mit172 and D4Mit23 (corrected $P =$

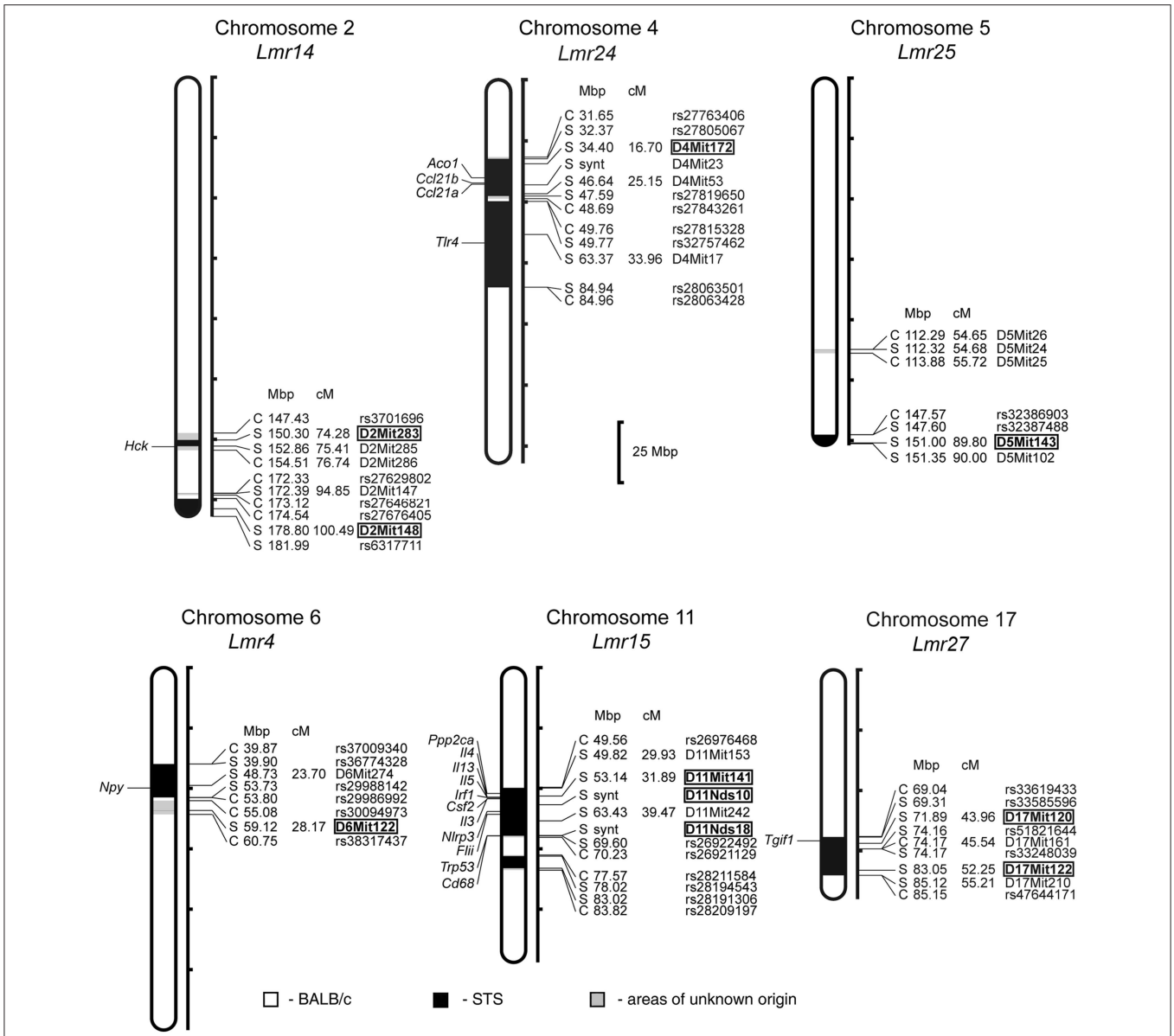

FIGURE 1 | Positions of the loci controlling parasite load in organs of the RC strain CcS-9. The regions derived from the BALB/c strain are indicated as white; the regions of the STS origin are indicated as black; and the areas of undetermined origin are gray. Only the markers that determine the boundaries between BALB/c and STS-derived segments, and the markers used for statistical calculations are shown. Boxes indicate the markers that exhibited significant *P*-values, corrected for genome-wide search. The genes that participate in the infection-related immune processes (16, 17, 20, 22, 33) and are located within the present *Lmr* loci are listed next to their positions at the chromosomes. Abbreviations indicate genes related to the response to *Leishmania* ssp.: *Hck*, hemopoietic cell kinase; *Aco1*, aconitase 1; *Ccl21a*, chemokine (C-C motif) ligand 21A (serine); *Ccl21b,* chemokine (C-C motif) ligand 21B (leucine), *Tlr4*, toll-like receptor 4; *Npy*, neuropeptide Y; *Ppp2ca*, protein phosphatase 2, formerly 2A; catalytic subunit, alpha isoform; *Il3*, interleukin 3; *Il4*, interleukin 4; *Il13*, interleukin 13; *Irf1*, interferon regulatory factor 1; *Csf2*, colony stimulating factor 2 (granulocyte-macrophage); *Nlrp3*, NLR family, pyrin domain containing 3; *Flii*, flightless I actin binding protein; *Trp53*, transformation related protein 53; Cd68, CD68 antigen; *Tgif1*, TGFB-induced factor homeobox 1.

0.00201 and 0.0226, respectively), level of IFNγ in serum—with D4Mit53 (corrected $P = 0.000692$, 8.08% of variance explained), and level of IL-4 in serum—with D4Mit53 (corrected $P = 0.0132$, 5.72% of variance explained). The STS allele of these markers was responsible for lower level of IFNγ and IL-4 in serum, but with larger splenomegaly and larger skin lesions.

In addition to the novel locus, we detected new functions of previously mapped loci (**Table 7**). *Lmr14* was detected in F_2 hybrids between CcS-16 and BALB/c. It was previously shown that it controlled hepatomegaly, splenomegaly, level of IgE and IFNγ in serum, and also infiltration of eosinophils to inguinal lymph nodes (33, 40, 44, 45). The present study revealed the role of *Lmr14* (marker D2Mit283) in control of level of IL-13 in

TABLE 2 | Interactions that control parasite load in lymph nodes of CcS-9 derived F_2 hybrids of both sexes.

Lymph nodes		D5Mit143 (*Lmr25*)											
		CC			CS			SS			*P*-value	corr. *P*	% of explained variance
D2Mit283 (*Lmr14*)	CC	$n = 20$	**2.34**	5.45 ± 0.22	$n = 21$	**1.33**	4.89 ± 0.21	$n = 18$	**1.20**	4.79 ± 0.23	0.00044	**0.0314**	**2.1**
	CS	$n = 31$	**1.03**	4.64 ± 0.19	$n = 57$	**2.19**	5.39 ± 0.13	$n = 30$	**1.93**	5.27 ± 0.18			
	SS	$n = 24$	**1.09**	4.69 ± 0.20	$n = 32$	**1.60**	5.08 ± 0.17	$n = 20$	**2.26**	5.42 ± 0.21			
Lymph nodes		**D2Mit283 (*Lmr14*)**											
		CC			**CS**			**SS**			***P*-value**	**corr. *P***	**% of explained variance**
D4Mit172 (*Lmr24*)	CC	$n = 15$	**1.25**	4.83 ± 0.27	$n = 22$	**1.78**	5.18 ± 0.21	$n = 16$	**1.70**	5.14 ± 0.24	0.00034	**0.0247**	**2.0**
	CS	$n = 28$	**1.47**	4.99 ± 0.18	$n = 59$	**2.05**	5.32 ± 0.12	$n = 36$	**1.01**	4.61 ± 0.17			
	SS	$n = 16$	**2.03**	5.31 ± 0.23	$n = 37$	**1.20**	4.79 ± 0.18	$n = 24$	**2.29**	5.43 ± 0.20			

Means, standard error of mean (SE) and P-values were calculated by analysis of variance. The letters C and S indicate the BALB/c or STS allele, respectively; n indicates the number of mice in each group. Transformed means ± SE are shown next to non-transformed mean values in bold. The values of concentration of parasite DNA (ng/ml) in total DNA isolated from lymph nodes were transformed to obtain normal distribution: a natural logarithm of (value × 100). Only P-values significant after correction for genome-wide significance are shown.

TABLE 3A | The *Lmr15* controls parasite load in lymph nodes of CcS-9 derived females.

Lymph nodes	CC			CS			SS					
Marker	***n***	**Mean**	**Transformed mean ± SE**	***n***	**Mean**	**Transformed mean ± SE**	***n***	**Mean**	**Transformed mean ± SE**	***P*-value**	**corr. *P***	**% of Explained variance**
D11Mit141 (*Lmr15*) both sexes	56	**2.14**	5.37 ± 0.15	127	**1.56**	5.05 ± 0.10	70	**1.11**	4.71 ± 0.13	0.00419	0.157	2.56
D11Mit141 (*Lmr15*) females	36	**1.44**	4.97 ± 0.17	69	**1.02**	4.62 ± 0.13	33	**0.45**	3.80 ± 0.18	0.000014	**0.00102**	**6.38**
D11Mit141 (*Lmr15*) males	20	**3.18**	5.76 ± 0.23	58	**3.58**	5.88 ± 0.14	37	**2.99**	5.70 ± 0.17	0.695	6.890	0.29
D11Nds10 (*Lmr15*) both sexes	58	**2.22**	5.40 ± 0.16	129	**1.54**	5.03 ± 0.09	66	**1.13**	4.73 ± 0.13	0.00395	0.149	3.01
D11Nds10 (*Lmr15*) females	36	**1.58**	5.06 ± 0.18	70	**0.99**	4.60 ± 0.13	32	**0.45**	3.80 ± 0.18	0.0000092	**0.000696**	**7.42**
D11Nds10 males	22	**3.12**	5.74 ± 0.26	59	**3.11**	5.74 ± 0.15	34	**3.37**	5.82 ± 0.19	0.935	7.811	2.5
D11Nds18 (*Lmr15*) both sexes	51	**1.67**	5.12 ± 0.16	136	**1.79**	5.19 ± 0.08	66	**1.08**	4.69 ± 0.12	0.00281	0.112	2.95
D11Nds18 (*Lmr15*) females	33	**1.21**	4.80 ± 0.16	73	**0.97**	4.58 ± 0.11	32	**0.50**	3.91 ± 0.16	0.00018	**0.0101**	**4.2**
D11Nds18 (*Lmr15*) males	18	**1.86**	5.23 ± 0.37	63	**2.61**	5.56 ± 0.19	34	**4.13**	6.02 ± 0.2	0.000175	**0.0106**	**16.7**

Means, standard error of mean (SE) and P-values were calculated by analysis of variance. The numbers in bold give the average non-transformed values. The letters C and S indicate the BALB/c or STS allele, respectively; n indicates the number of mice in each group. The values of concentration of parasite DNA (ng/ml) in total DNA isolated from lymph nodes were transformed to obtain normal distribution: a natural logarithm of (value × 100). Only P-values significant after correction for genome-wide significance are shown.

serum (corrected $P = 0.0146$, 10.76% of variance explained). The STS allele of this marker was linked with higher level of IL-13 in serum. *Lmr15* was detected in F_2 hybrids between CcS-16 and BALB/c and it influences hepatomegaly, IFNγ level in serum and infiltration of eosinophils in inguinal lymph nodes (33, 40, 45). The present study revealed several additional effects of *Lmr15*:

TABLE 3B | The *Lmr14* and *Lmr27* control parasite load in lymph nodes of CcS-9 derived males.

Lymph nodes	CC			CS			SS					
Marker	***n***	**Mean**	**Transformed mean ± SE**	***n***	**Mean**	**Transformed mean ± SE**	***n***	**Mean**	**Transformed mean ± SE**	***P* value**	**corr. *P***	**% of explained variance**
D2Mit148 (*Lmr14*) both sexes	78	**1.72**	5.15 ± 0.13	121	**1.61**	5.08 ± 0.11	54	**1.68**	5.13 ± 0.15	0.908	7.725	0.93
D2Mit148 (*Lmr14*) females	43	**0.83**	4.41 ± 0.14	69	**0. 89**	4.49 ± 0.12	26	**0. 67**	4.20 ± 0.18	0.411	5.220	0.39
D2Mit148 (*Lmr14*) males	35	**3.04**	5.72 ± 0.19	52	**3.11**	5.74 ± 0.14	28	**16.20**	7.39 ± 0.39	0.000616	**0.03095**	**10.5**
D17Mit122 (*Lmr27*) both sexes	74	**1.61**	5.08 ± 0.12	142	**1.42**	4.96 ± 0.10	37	**2.04**	5.32 ± 0.19	0.209	3.399	2.28
D17Mit122 (*Lmr27*) females	42	**0.89**	4.49 ± 0.15	77	**0.77**	4.35 ± 0.11	19	**0.89**	4.48 ± 0.21	0.678	6.813	0.17
D17Mit122 (*Lmr27*) males	32	**3.44**	5.84 ± 0.18	65	**2.84**	5.65 ± 0.13	18	**15.56**	7.35 ± 0.36	0.000206	**0.0118**	**15.3**

Means, standard error of mean (SE) and P-values were calculated by analysis of variance. The numbers in bold give the average non-transformed values. The letters C and S indicate the BALB/c or STS allele, respectively; n indicates the number of mice in each group. The values of concentration of parasite DNA (ng/ml) in total DNA isolated from lymph nodes were transformed to obtain normal distribution: a natural logarithm of (value × 100). Only P-values significant after correction for genome-wide significance are shown.

TABLE 4 | Interactions that control parasite load in lymph nodes of CcS-9 derived males.

Lymph nodes		D11Nds10 (*Lmr15*)										
		CC			CS			SS			***P* value**	**corr. *P***	**% of explained variance**
D17Mit122 (*Lmr27*)	CC	*n* = 7	**2.50**	5.52 ± 0.31	*n* = 12	**3.22**	5.77 ± 0.24	*n* = 13	**5.06**	6.23 ± 0.23	0.000339	**0.0261**	**6.0**
	CS	*n* = 11	**3.97**	5.98 ± 0.25	*n* = 37	**2.92**	5.68 ± 0.14	*n* = 17	**2.00**	5.30 ± 0.20			
	SS	*n* = 4	**4.68**	6.15 ± 0.41	*n* = 10	**52.10**	8.56 ± 0.26	*n* = 4	**15.53**	7.35 ± 0.41			
Lymph nodes		**D6Mit122 (*Lmr4*)**										
		CC			**CS**			**SS**			***P*-value**	**corr. *P***	**% of explained variance**
D17Mit122 (*Lmr27*)	CC	*n* = 10	**5.21**	6.26 ± 0.26	*n* = 13	**2.76**	5.62 ± 0.23	*n* = 9	**2.84**	5.65 ± 0.23	0.0000467	**0.00427**	**7.4**
	CS	*n* = 17	**2.35**	5.46 ± 0.20	*n* = 35	**4.26**	6.05 ± 0.14	*n* = 13	**2.31**	5.44 ± 0.20			
	SS	*n* = 5	**73.38**	8.90 ± 0.37	*n* = 9	**5.65**	6.34 ± 0.28	*n* = 4	**9.13**	6.82 ± 0.41			

Means, standard error of mean (SE) and P-values were calculated by analysis of variance. The letters C and S indicate the BALB/c or STS allele, respectively; n indicates the number of mice in each group. Transformed means ± SE are shown next to non-transformed mean values in bold. The values of concentration of parasite DNA (ng/ml) in total DNA isolated from lymph nodes were transformed to obtain normal distribution: a natural logarithm of (value × 100). Only P-values significant after correction for genome-wide significance are shown.

control of splenomegaly (markers D11Mit141 and D11Nds10, corrected $P = 0.0313$ and 0.0235, respectively, 9.16 and 11.80% of variance explained), lesion size in week 8 after infection (markers D11Nds10 and D11Nds18, corrected $P = 0.0282$ and 0.0424, respectively, 9.37 and 4.21% of variance explained), kinetics of development of skin lesions (markers D11Nds18 and D11Nds10, corrected $P = 0.0282$ and 0.0406, respectively) and level of IgE in serum (markers D11Mit141, D11Mit242, and D11Nds10, corrected $P = 0.0000460$, 0.0148 and 0.0000129, respectively, 10.93, 6.23, and 13.00% of variance explained). The STS allele of these markers was linked with lower level of IgE in serum and smaller splenomegaly and skin lesions.

In addition, we detected two interactions between loci which control different parameters of the infection (**Table 8**). Interaction between markers D11Nds10 (*Lmr15*) and D16Mit19 (*Lmr18*) controlled lesion size in 7th week after infection. BALB/c

TABLE 5 | The *Lmr15* controls parasite load in liver of CcS-9 derived F_2 hybrids of both sexes.

Liver	CC			CS			SS					
Marker	***n***	**Mean**	**Transformed mean ± SE**	***n***	**Mean**	**Transformed mean ± SE**	***n***	**Mean**	**Transformed mean ± SE**	***P*-value**	**corr. *P***	**% of explained variance**
D11Mit141 (*Lmr15*)	56	**3.43**	5.84 ± 0.11	128	**2.90**	5.67 ± 0.07	69	**1.87**	5.23 ± 0.10	0.000061	**0.00373**	**8.6**
D11Nds10 (*Lmr15*)	58	**3.36**	5.82 ± 0.12	130	**3.06**	5.72 ± 0.08	65	**1.87**	5.23 ± 0.10	0.000015	**0.00106**	**9.6**

Means, standard error of mean (SE) and P-values were calculated by analysis of variance. The numbers in bold give the average non-transformed values. The letters C and S indicate the BALB/c or STS allele, respectively; n indicates the number of mice in each group. The values of concentration of parasite DNA (ng/ml) in total DNA isolated from liver were transformed to obtain normal distribution: a natural logarithm of (value × 100). Only P-values significant after correction for genome-wide significance are shown.

TABLE 6 | Interactions that control parasite load in liver of CcS-9 derived F_2 hybrids of both sexes.

Liver		D2Mit283 (*Lmr14*)										
		CC			CS			SS			***P* value**	**corr. *P***	**% of explained variance**
D17Mit120 (*Lmr27*)	CC	$n = 17$	**1.99**	5.29 ± 0.19	$n = 29$	**2.75**	5.62 ± 0.15	$n = 27$	**3.29**	5.80 ± 0.15			
	CS	$n = 31$	**3.56**	5.87 ± 0.14	$n = 63$	**3.08**	5.73 ± 0.10	$n = 44$	**1.78**	5.18 ± 0.12	0.00024	**0.0184**	**6.9**
	SS	$n = 12$	**1.91**	5.25 ± 0.22	$n = 25$	**2.67**	5.59 ± 0.15	$n = 5$	**3.44**	5.84 ± 0.35			
Liver		**D5Mit143 (*Lmr25*)**										
		CC			**CS**			**SS**			***P*-value**	**corr. *P***	**% of explained variance**
	CC	$n = 30$	**3.17**	5.76 ± 0.13	$n = 28$	**3.93**	5.97 ± 0.14	$n = 16$	**1.57**	5.05 ± 0.19			
D17Mit122 (*Lmr27*)	CS	$n = 36$	**2.80**	5.63 ± 0.13	$n = 64$	**2.98**	5.70 ± 0.10	$n = 41$	**2.54**	5.54 ± 0.12	0.00096	**0.0640**	**6.2**
	SS	$n = 9$	**2.21**	5.40 ± 0.26	$n = 18$	**2.02**	5.31 ± 0.20	$n = 11$	**3.85**	5.95 ± 0.23			

Means, standard error of mean (SE) and P-values were calculated by analysis of variance. The letters C and S indicate the BALB/c or STS allele, respectively; n indicates the number of mice in each group. Transformed means ± SE are shown next to non-transformed mean values in bold. The values of concentration of parasite DNA (ng/ml) in total DNA isolated from liver were transformed to obtain normal distribution: a natural logarithm of (value × 100). Only P-values significant after correction for genome-wide significance are shown.

homozygosity at *Lmr15* synergizes with BALB/c homozygosity at *Lmr18* in enlarging size of skin lesions (corrected $P = 0.044$, 6.71% of variance explained). Interaction between marker D4Mit53 (*Lmr24*) and D6Mit122 (*Lmr4*) controlled level of IL-10 in serum. STS homozygosity at *Lmr24* synergized with STS homozygosity at *Lmr4* in increasing of level of IL-10 in serum (corrected $P = 0.0156$, 9.61% of variance explained. The control of organ pathology and immune response in CcS-9 was not sex dependent.

The CcS-16 Strain: One Novel *Lmr* Locus Confirmed, Control of Parasite Load Detected for Two Additional Loci

Only female mice were used in the study with F_2 hybrids between the BALB/c parental mouse strain and the RC strain CcS-16, because previous analysis of response to *L. major* in different RC strains (34) showed no significant dependence on sex in CcS-16 mice (in contrast to the strain CcS-9 described above). We confirmed a presence of a novel parasite controlling cluster *Lmr4* (**Figures 1**, **2**; **Table 9**) on chromosome 6, which influences parasite load in lymph nodes. A homozygous BALB/c (CC) allele in the *Lmr4* linked to D6Mit48 was responsible for higher parasite load (corrected $P = 0.017$, 4.95% of variance explained) (**Table 9**). The effect of *Lmr13* at the chromosome 18 linked to D18Mit40 on parasite load in liver was opposite to that of *Lmr4*: the STS allele (SS) was responsible for higher parasite load (corrected $P = 0.00056$, 10.4% of variance explained) (**Table 9**). Parasite load in liver controls also an interaction between *Lmr14* linked to D2Nds3 with *Lmr13* linked to D18Mit120 (suggestive linkage, corrected $P = 0.0777$, 4.8% of explained variance) (**Table 10**). Two genotype combinations determine highest parasite load: STS (SS) homozygotes in *Lmr13* with BALB/c (CC) homozygotes in *Lmr14*, and STS (SS) homozygotes in *Lmr14* with BALB/c (CC) homozygotes in *Lmr13*. A homozygous STS allele of *Lmr13* linked to the markers D18Mit35 (corrected $P = 0.0073$, 4.85% of variance explained) and D18Mit120 (corrected $P = 0.019$, 5.18% of variance explained) determined the higher load of parasites in spleen (**Table 9**). Parasite load in spleen was also controlled by *Lmr13* linked to D18Mit49 in interaction with *Lmr11* linked to D3Mit11 (corrected $P = 0.0295$, 3.4% of variance explained).

TABLE 7 | Loci controlling organ pathology and immunological parameters in *Leishmania major*-infected F2 hybrids between CcS-9 and BALB/c.

Phenotype	Locus	Marker	Genotype						*P*-value	corr. *P*	% of explained variance
			CC		CS		SS				
Splenomegaly	*Lmr15*	D11Mit141	**17.86** ± 0.74 (*n* = 56)		**17.29** ± 0.42 (*n* = 128)		**14.86** ± 0.57 (*n* = 70)		0.00066	**0.0313**	**9.16**
		D11Nds10	**18.07** ± 0.73 (*n* = 58)		**17.18** ± 0.42 (*n* = 130)		**14.77** ± 0.59 (*n* = 66)		0.00047	**0.0235**	**11.80**
	Lmr24	D4Mit23	**15.71** ± 0.64 (*n* = 57)		**15.83** ± 0.45 (*n* = 122)		**18.17** ± 0.54 (*n* = 75)		0.00093	**0.0426**	**4.76**
Hepatomegaly	*Lmr15*	D11Nds18	**64.02** ± 1.03 (*n* = 51)		**63.05** ± 0.60 (*n* = 136)		**58.50** ± 0.90 (*n* = 66)		0.000029	**0.00191**	**9.76**
Lesion size (in 7th week after infection)	*Lmr15*	D11Mit141	**83.68** ± 4.92 (*n* = 56)		**69.72** ± 3.00 (*n* = 128)		**55.68** ± 4.23 (*n* = 70)		0.000095	**0.00555**	**8.64**
		D11Mit242	**84.81** ± 5.00 (*n* = 51)		**69.18** ± 2.85 (*n* = 139)		**56.23** ± 4.00 (*n* = 64)		0.000044	**0.00274**	**7.55**
		D11Nds18	**90.07** ± 5.55 (*n* = 51)		**69.47** ± 2.88 (*n* = 137)		**55.87** ± 3.93 (*n* = 66)		0.0000035	**0.000269**	**10.06**
		D11Nds10	**86.07** ± 4.64 (*n* = 58)		**68.63** ± 2.91 (*n* = 130)		**55.25** ± 4.36 (*n* = 66)		0.000010	**0.000722**	**12.04**
Lesion size (in 8th week after infection)	*Lmr15*	D11Nds10	**101.56** ± 5.62 (*n* = 58)		**88.47** ± 3.25 (*n* = 130)		**73.30** ± 4.95 (*n* = 66)		0.00058	**0.0282**	**9.37**
		D11Nds18	**100.93** ± 5.11 (*n* = 51)		**87.94** ± 3.18 (*n* = 137)		**76.31** ± 4.36 (*n* = 66)		0.00093	**0.0424**	**4.21**
Lesion size (kinetics)	*Lmr15*	D11Nds18	**32.77**	5.72 ± 0.13 (*n* = 51)	**28.37**	5.33 ± 0.08 (*n* = 137)	**25.54**	5.05 ± 0.11 (*n* = 66)	0.00060	**0.0282**	–
		D11Nds10	**31.33**	5.60 ± 0.12 (*n* = 58)	**29.09**	5.39 ± 0.08 (*n* = 130)	**24.91**	4.99 ± 0.11 (*n* = 66)	0.00090	**0.0406**	–
	Lmr24	D4Mit172	**27.19**	5.21 ± 0.13 (*n* = 53)	**26.40**	5.14 ± 0.08 (*n* = 124)	**32.92**	5.74 ± 0.11 (*n* = 77)	0.000032	**0.00201**	–
		D4Mit23	**26.97**	5.19 ± 0.12 (*n* = 57)	**26.87**	5.18 ± 0.08 (*n* = 122)	**32.38**	5.69 ± 0.11 (*n* = 75)	0.00047	**0.0226**	–
Level of IgE in serum	*Lmr15*	D11Mit141	**35.09**	3.12 ± 0.10 (*n* = 56)	**25.26**	2.81 ± 0.07 (*n* = 127)	**15.29**	2.39 ± 0.09 (*n* = 70)	0.00000052	**0.0000460**	**10.93**
		D11Mit242	**33.89**	3.09 ± 0.11 (*n* = 51)	**25.78**	2.83 ± 0.07 (*n* = 138)	**17.70**	2.51 ± 0.10 (*n* = 64)	0.00028	**0.0148**	**6.23**
		D11Nds10	**36.94**	3.17 ± 0.11 (*n* = 58)	**25.96**	2.84 ± 0.07 (*n* = 129)	**13.72**	2.31 ± 0.11 (*n* = 66)	0.00000013	**0.0000129**	**13.00**
Level of INFγ in serum	*Lmr24*	D4Mit53	**13.20**	2.81 ± 0.14 (*n* = 59)	**5.93**	2.04 ± 0.09 (*n* = 120)	**6.58**	2.12 ± 0.12 (*n* = 74)	0.0000097	**0.000692**	**8.08**
Level of IL-4 in serum	*Lmr24*	D4Mit53	**1.79**	0.47 ± 0.06 (*n* = 59)	**1.20**	0.17 ± 0.05 (*n* = 120)	**1.26**	0.21 ± 0.06 (*n* = 74)	0.00025	**0.0132**	**5.72**
		D11Nds10	**1.65**	0.42 ± 0.07 (*n* = 58)	**1.48**	0.34 ± 0.04 (*n* = 129)	**1.10**	0.09 ± 0.06 (*n* = 66)	0.00046	**0.0230**	**6.73**
Level of IL-13 in serum	*Lmr14*	D2Mit283	**518.66**	22.77 ± 1.18 (*n* = 44)	**551.08**	23.48 ± 0.84 (*n* = 82)	**830.30**	28.81 ± 1.24 (*n* = 57)	0.00027	**0.0146**	**10.76**

Only linkages significant at whole genome level are given. In order to obtain normal distribution required for analysis of variance, the following transformations were used: lesion size (mm^2)—power of 0.5; IgE in serum (μg/ml)—power of 0.32; IFN-γ in serum (ng/ml)—power of 0.4; IL-4 in serum (ng/ml)—Box-Cox transformation: [(x^(−0.720642))−1]/(−0.720642), IL-13 in serum (pg/ml)—power of 0.5. The numbers in bold give the average non-transformed values. C and S indicate the presence of BALB/c and STS allele, respectively; n indicates number of mice.

Highest parasite load was observed in combination of STS (SS) homozygotes in *Lmr13* and BALB/c (CC) homozygotes in *Lmr11* (**Table 10**).

DISCUSSION

Parasite load and dissemination in visceral organs of infected host belong to the most important parameters in leishmaniasis. Elucidation of host determinants of parasite control could help to better understand these critical disease mechanisms. We have therefore addressed the following questions: genetic control of parasite load in organs after *L. major* infection, analysis of relationship among parasite-controlling genes and the genes controlling organ pathology, and role of sex in control of parasite load. We defined two new genetic loci controlling parasite load (*Lmr24* and *Lmr27*) and described that

TABLE 8 | Interactions between loci that control lesion size and level of IL-10 in serum in *Leishmania major*-infected F_2 hybrids between CcS-9 and BALB/c.

Lesion size in 7th week after infection		**D16Mit19 (*Lmr18*)**								
		CC		**CS**		**SS**		***P*-value**	**corr. *P***	**% of explained variance**
D11Nds10 (*Lmr15*)	CC	$n = 99$	111.45 ± 10.05	$n = 30$	73.89 ± 5.61	$n = 19$	66.25 ± 6.88	0.00064	**0.044**	**6.71**
	CS	$n = 32$	59.48 ± 5.32	$n = 66$	68.33 ± 3.75	$n = 32$	76.07 ± 5.40			
	SS	$n = 20$	63.07 ± 6.80	$n = 36$	55.56 ± 5.13	$n = 10$	51.56 ± 9.47			

Level of IL-10 in serum		**D4Mit53 (*Lmr24*)**										
		CC			**CS**			**SS**			***P* value**	**corr. *P***	**% of explained variance**
D6Mit122 (*Lmr4*)	CC	$n = 11$	**1.52**	0.32 ± 0.03	$n = 24$	**2.10**	0.47 ± 0.02	$n = 15$	**2.01**	0.45 ± 0.02	0.00020	**0.0156**	**9.61**
	CS	$n = 27$	**2.12**	0.47 ± 0.02	$n = 44$	**1.94**	0.43 ± 0.01	$n = 31$	**1.92**	0.43 ± 0.02			
	SS	$n = 10$	**1.87**	0.42 ± 0.03	$n = 20$	**1.93**	0.43 ± 0.02	$n = 15$	**2.15**	0.47 ± 0.02			

In order to obtain normal distribution required for analysis of variance, the following transformation was used for level of IL-10 in serum (ng/ml) – [(x^(−1.372544)]-1)/(−1.372544). C and S indicate the presence of BALB/c and STS allele, respectively; n indicates number of mice. Bold: non-transformed values.

six previously mapped loci (*Lmr4*, *Lmr11*, *Lmr13*, *Lmr14*, *Lmr15*, and *Lmr25*) also control parasite load. This enabled us to show a genetic network controlling interaction between parasite and mammalian host.

Control of Parasite Load in Organs: Multiple Genes and Distinct Organ Specificities That Operate in a Network With Many Gene-Gene Interactions

Visceral leishmaniasis is a life-threatening disease (9, 13) and hence understanding parasite spread to organs is essential. Although many of its host, vector, and parasite determinant have been described (46), the role of the host in control of parasite visceralization remains largely unknown. The importance of host determinants that can limit leishmaniasis to a cutaneous form or allow progressive visceral pathology is stressed by the observation that even though *L. major* causes mostly only cutaneous pathology in human, cases of visceralization are also known (31, 32). We have therefore studied parasite load after *L. major* infection in three organs: lymph nodes, spleen and liver, and found that parasite load in each tested organ is controlled by multiple genes. In this work, we report six loci (*Lmr4*, *Lmr14*, *Lmr15*, *Lmr24*, *Lmr25,* and *Lmr27*) that control parasite load in lymph nodes of the strains CcS-9 and CcS-16 (**Table 11**). *Lmr4*, *Lmr14,* and *Lmr15* can operate independently from other genes (main effect loci), whereas *Lmr24*, *Lmr25,* and *Lmr27* operate only in interaction with other loci (**Figure 3**). Previously, we described another locus, *Lmr20,* which influences parasite load in lymph nodes of the strain CcS-11 (21).

We have found two loci that control parasite load in spleen of the strain CcS-16: *Lmr13* and *Lmr11* (**Table 11B**). *Lmr13* operates as main effect locus, role of *Lmr11* is visible only in interaction with *Lmr13* (**Tables 9**, **10**). No locus controlling parasite load in spleen was detected in the strain CcS-9 although spleens of this strain contain parasites (47). This might be caused by the fact that parasite load in spleen in the strain CcS-9 is controlled by multiple weak loci and our experiments lacked the power to detect them. Thus, with the previously detected *Lmr5* controlling parasites in spleen in CcS-11 (21), there are three different loci controlling *L. major* load in spleen. Parasite load in liver is controlled by two main effect loci *Lmr13* and *Lmr15*, operating in the strains CcS-16 and CcS-9, respectively, and three loci *Lmr14, Lmr25* (suggestive linkage) and *Lmr27* (in the strain CcS-9), whose effect is observed only in interaction.

Comparison of loci that control parasite burden in organs indicates organ specific control of parasite load, where dissemination of parasites to lymph nodes, spleen and liver were controlled by distinct sets of loci that only partly overlaps (**Table 11**). Some loci, such as *Lmr13*, *Lmr14*, *Lmr15*, *Lmr25,* and *Lmr27* determined parasite load in two different organs, effects of others were limited to only one organ. Organ specific control was described also in candidate gene based studies of parasite burden after infection with *L. donovani*, where genes *Slc11a1*, *H2* (25) and *Lyst* (24) control parasite load in spleen, whereas *Slc11a1* (26), *H2* (27, 28) and *Ir2* (28) determine parasite load in liver. Genome-wide studies with *L. tropica* infection showed that parasite load in lymph nodes are determined by *Ltr1* and *Ltr4*, in spleen by *Ltr3* and *Ltr6*, and in liver by *Ltr2*, *Ltr4,* and *Ltr8* (23) (**Table 1**). On the mechanistic level, organ-specific genetic control might be based on organ-specific immune response to *Leishmania* parasites (48). Some of the loci that determine parasite load in *L. donovani* (MHOM/SD/62/3S) and *L. tropica* (MHOM/1999/TR/SU23) overlap with the *Lmr* loci: *Lmr13* that controls parasite load in liver and spleen co-localize with *Ltr8* influencing parasite load in liver (23), *Lmr14* (suggestive linkage) influencing in interaction with *Lmr13* parasite load

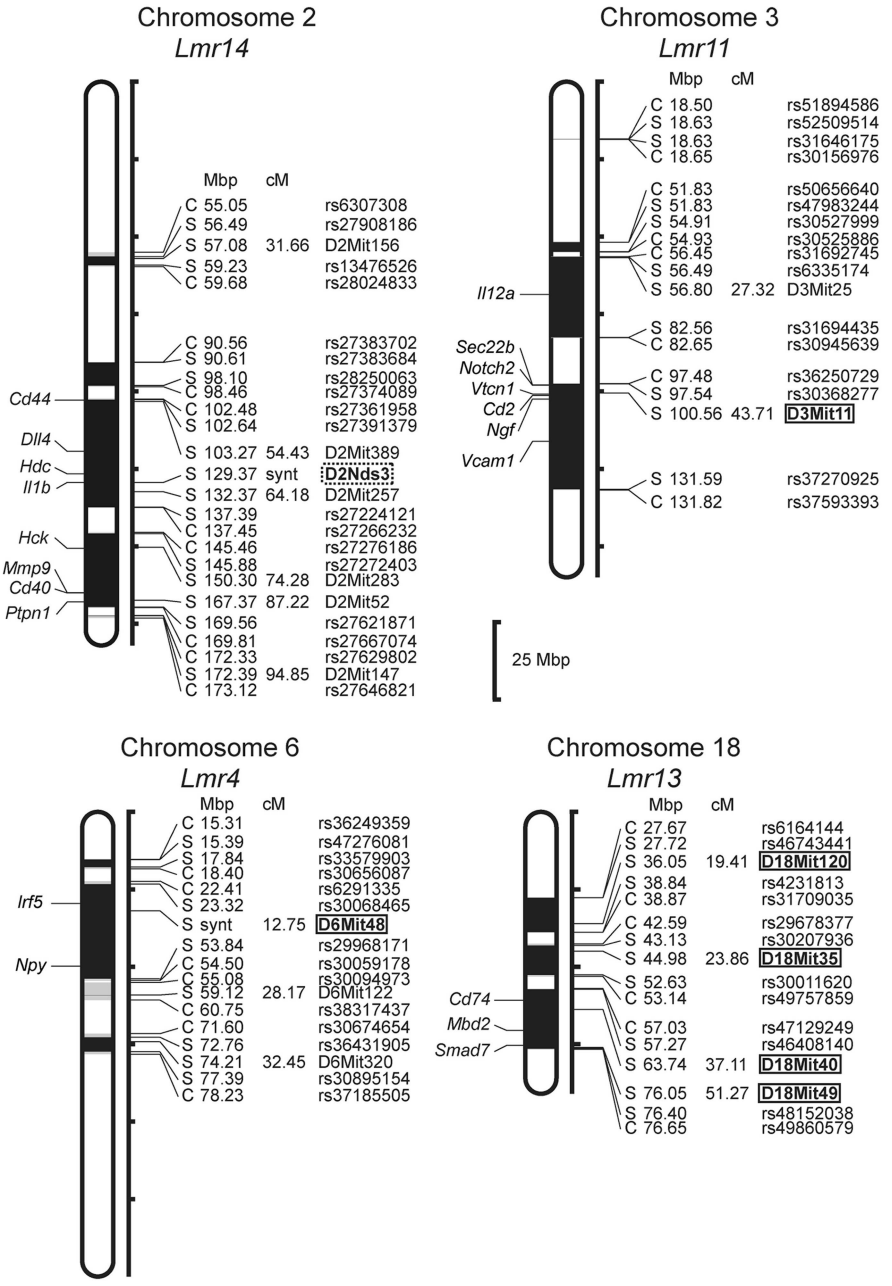

FIGURE 2 | Positions of the loci controlling parasite load in organs of the RC strain CcS-16. The regions derived from the BALB/c strain are indicated as white; the regions of the STS origin are indicated as black; and the areas of undetermined origin are gray. Only the markers that determine the boundaries between BALB/c and STS-derived segments, and the markers used for statistical calculations are shown. Boxes indicate the markers that exhibited significant *P*-values, corrected for genome-wide search, dotted box—suggestive linkage. The genes that participate in the infection-related immune processes (16, 17, 20, 22, 23) and are located within the present *Lmr* loci are listed next to their positions at the chromosomes. Abbreviations indicate genes related to the response to *Leishmania* ssp.: *Cd44*, CD44 antigen; *Dll4*, delta like canonical Notch ligand 4; *Hdc*, histidine decarboxylase; *Il1b*, interleukin 1 beta; *Hck*, hemopoietic cell kinase; *Mmp9*, matrix metallopeptidase 9; *Cd40*, CD40 antigen; *Ptpn1*, protein tyrosine phosphatase, non-receptor type 1; *Il12a*, interleukin 12a; *Sec22b*, SEC22 homolog B, vesicle trafficking protein; *Notch2*, notch 2; *Vtcn1*, V-set domain containing T cell activation inhibitor 1; *Cd2*, CD2 antigen; *Ngf,* nerve growth factor; *Vcam1*, vascular cell adhesion molecule 1; *Irf5*, interferon regulatory factor 5; *Npy*, neuropeptide Y; *Cd74*, CD74 antigen [invariant polypeptide of major histocompatibility complex, class II antigen-associated)], *Mbd2*, methyl-CpG binding domain protein 2; *Smad7*, SMAD family member 7.

in liver overlaps with *Ir2* (28) and *Ltr2* (23) that control parasite load in liver after infection with *L. donovani* and *L. tropica*, respectively. Thus, similarly as *H2* or *Slc11a1*, some parasite-controlling *Lmr* loci might affect several pathogens, probably reflecting immune responses effective against groups of infectious agents.

TABLE 9 | The *Lmr4* controls parasite load in lymph nodes, and *Lmr13* controls parasite load in liver and spleen of CcS-16 derived F_2 hybrids.

Marker	CC			CS			SS			*P*-value	corr. *P*	% of explained variance
	n	Mean	Transformed mean ± SE	*n*	Mean	Transformed mean ± SE	*n*	Mean	Transformed mean ± SE			
Lymph nodes												
D6Mit48 (*Lmr4*)	104	**1.98**	5.29 ± 0.12	230	**1.16**	4.75 ± 0.09	109	**1.12**	4.72 ± 0.12	0.00032	**0.017**	**4.95**
Liver												
D18Mit40 (*Lmr13*)	92	**0.52**	3.96 ± 0.12	178	**0.43**	3.75 ± 0.09	71	**1.03**	4.63 ± 0.16	0.0000078	**0.00056**	**10.4**
Spleen												
D18Mit35 (*Lmr13*)	136	**0.52**	3.95 ± 0.13	274	**0.87**	4.46 ± 0.09	133	**1.07**	4.67 ± 0.12	0.00013	**0.0073**	**4.85**
D18Mit120 (*Lmr13*)	133	**0.52**	3.95 ± 0.13	284	**0.87**	4.47 ± 0.09	130	**0.99**	4.59 ± 0.12	0.00038	**0.019**	**5.18**

*Means, standard error of mean (SE) and P-values were calculated by analysis of variance. The letters C and S indicate the BALB/c or STS allele, respectively; n indicates the number of mice in each group. The numbers in bold give the average non-transformed values. The values of concentration of parasite DNA (ng/ml) in total DNA isolated from lymph nodes, liver and spleen were transformed to obtain normal distribution: a natural logarithm of a (value *100). Only P-values significant after correction for genome-wide significance are shown.*

TABLE 10 | Interactions that control parasite load in spleen and liver of CcS-16 derived F_2 hybrids.

Spleen		D3Mit11 (*Lmr11*)											
		CC			CS			SS			*P*-value	corr. *P*	% of explained variance
D18Mit49 (*Lmr13*)	CC	*n* = 27	**0.40**	3.68 ± 0.25	*n* = 78	**0.93**	4.53 ± 0.14	*n* = 37	**0.63**	4.14 ± 0.21			
	CS	*n* = 59	**0.78**	4.35 ± 0.17	*n* = 175	**0.83**	4.42 ± 0.10	*n* = 48	**0.84**	4.43 ± 0.18	0.00042	**0.0295**	**3.4**
	SS	*n* = 35	**1.45**	4.98 ± 0.21	*n* = 53	**0.59**	4.07 ± 0.17	*n* = 34	**0.99**	4.59 ± 0.22			
Liver		**D2Nds3 (*Lmr14*)**											
		CC			CS			SS			*P* value	corr. *P*	% of explained variance
	CC	*n* = 28	**0.48**	3.87 ± 0.18	*n* = 37	**0.49**	3.88 ± 0.15	*n* = 15	**0.80**	4.38 ± 0.24			
D18Mit120 (*Lmr13*)	CS	*n* = 38	**0.37**	3.61 ± 0.15	*n* = 99	**0.45**	3.81 ± 0.10	*n* = 41	**0.53**	3.96 ± 0.15	0.0012	**0.0777**	**4.8**
	SS	*n* = 15	**0.97**	4.58 ± 0.24	*n* = 41	**0.77**	4.35 ± 0.15	*n* = 22	**0.37**	3.62 ± 0.20			

*Means, standard error of mean (SE) and P-values were calculated by analysis of variance. The letters C and S indicate the BALB/c or STS allele, respectively; n indicates the number of mice in each group. Transformed means ± SE are shown next to mean non-transformed values in bold. The values of concentration of parasite DNA (ng/ml) in total DNA isolated from spleen and liver were transformed to obtain normal distribution: a natural logarithm of (a value*100). Only P-values significant after correction for genome-wide significance are shown.*

Relationship Between Control of Parasite Load, Organ Pathology, and Systemic Immune Response

We assessed relationship between parasite control, organ pathology, and systemic immune response (**Table 11**) and found a large heterogeneity in effects of controlling loci. Moreover, control of parasite load was linked with control of organ pathology in some loci, but not in others. Some loci, such as *Lmr13* and *Lmr14* carried by CcS-16, and *Lmr15* and *Lmr24* carried by CcS-9 controlled both parasite loads in organs, organ pathology and systemic immune response: *Lmr13* determined parasite load in spleen and liver, skin lesions (40), IgE in serum (44), TNFα in serum (45); *Lmr14* (CcS-16) influenced parasite load in liver (suggestive linkage), splenomegaly, hepatomegaly (40), IgE (44), IFNγ, IL-12, and TNFα in serum, and spontaneous proliferation of splenocytes from infected mice (45); *Lmr15* (CcS-9) controlled parasite load in lymph nodes and in liver, infiltration of eosinophils into lymph nodes (33), skin lesions, hepatomegaly, IL-4 and IgE in serum; and *Lmr24* controlled parasite load in lymph nodes, skin lesions, splenomegaly and IL-4, IL-10, and IFNγ in serum). *Lmr14* carried by CcS-9 determined parasite load in lymph nodes and in liver and systemic immune response (IL-13 in serum), but no organ pathology. *Lmr15* carried by CcS-16 did not influence parasite load, but controls hepatomegaly (40) and IFNγ in serum (45). Loci *Lmr14* and *Lmr15* might represent clusters of genes, their STS-derived segments in CcS-9 and CcS-16 overlap, but are not identical and they controlled different combinations of parameters in these strains (**Tables 11A**,**B**). Loci *Lmr4, Lmr11,* and *Lmr27* controlled parasite load in organs, but neither organ pathology nor systemic immune response (**Table 11**). Locus *Lmr25* controls parasite load in lymph nodes, infiltration of eosinophils to the lymph nodes,

TABLE 11A | Summary of loci that control parasite load detected in CcS-9 study.

Chr.	*Lmr* loci	Markers	Sex	Parasite control in organ	Response to parasite
2	*Lmr14*	D2Mit148			IL-13 in serum
		D2Mit283	M	Lymph nodes	Eosinophils in lymph nodes**#
2 and 4	Interaction, *Lmr14* and *Lmr24*	D2Mit283 D4Mit172		Lymph nodes	
2 and 5	Interaction, *Lmr14* and *Lmr25*	D2Mit283 D5Mit143		Lymph nodes	
2 and 17	Interaction, *Lmr14* and *Lmr27*	D2Mit283 D17Mit120		Liver	
4	*Lmr24*	D4Mit23 D4Mit53 D4Mit172			Skin lesion Splenomegaly IL-4, IFNγ in serum
4 and 6	Interaction, *Lmr4* and *Lmr24*	D4Mit53 D6Mit122			IL-10 in serum
5	*Lmr25*	D5Mit143			Eosinophils in lymph nodes**
5 and 17	Interaction, *Lmr25* and *Lmr27*	D5Mit143 D17Mit122		Liver*	
6 and 17	Interaction, *Lmr4* and *Lmr27*	D6Mit122 D17Mit122	M	Lymph nodes	
9	Interaction, *Lmr15* and *Lmr26*	D9Mit15 D11Nds10	M		Eosinophils in lymph nodes***
11	*Lmr15*	D11Nds18 D11Nds10 D11Mit141		Liver	Skin lesion Splenomegaly Hepatomegaly
			F	Lymph nodes	IL-4, IgE in serum
			M	Lymph nodes	
11 and 16	Interaction, *Lmr15* and *Lmr18*	D11Nds10 D16Mit19			Skin lesion
11 and 17	Interaction, *Lmr15* and *Lmr27*	D11Nds10 D17Mit122	M	Lymph nodes	
17	*Lmr27*	D17Mit122	M	Lymph nodes	

**Suggestive linkage.*
***Data from Slapničková et al. (33).*
****Interaction between sex and marker was not significant.*
 # Significant only in males from the cross CcS-9 × BALB/c.

TABLE 11B | Summary of loci that control parasite load and response to infection in CcS-16.

Chr.	*Lmr* loci	Markers	Parasite control in organ	Immune response
2	*Lmr14*	D2Mit52 D2Mit102 D2Mit283 D2Mit389 D2MitNds3		Splenomegaly Hepatomegaly Spontaneous proliferation of splenocytes from infected mice IFNγ in serum
2 and 10	Interaction, *Lmr5,* and *Lmr14*	D2Mit102 D10Mit103 D2Mit389		IL-12, IgE in serum
2 and 16	Interaction, *Lmr12* and *Lmr14*	D2Mit283 D16Mit126		TNFα in serum
2 and 18	Interaction, *Lmr13* and *Lmr14*	D2Nds3 D18Mit120	Liver*	TNFα in serum
3 and 18	Interaction, *Lmr11* and *Lmr13*	D3Mit11 D18Mit49	Spleen	
6	*Lmr4*	D6Mit48	Lymph nodes	
10 and 16	Interaction, *Lmr12* and *Lmr19*	D10Mit65 D16Mit126		Spontaneous proliferation of splenocytes from infected mice
11	*Lmr15*	D11Mit37 D11Mit 139 D11Mit 242		Hepatomegaly IFNγ in serum
16	*Lmr12*	D16Mit126		IL-4, IgE in serum
18	*Lmr13*	D18Mit35 D18Mit40 D18Mit120	Spleen Liver	Lesion size IgE in serum

*Table summarizes data from Vladimirov et al. (40), Badalová et al. (44), and Havelková et al. (45) and this study. *Suggestive linkage.*

but no skin or visceral pathology. Locus *Lmr18* influences only skin lesions. *Lmr26* controls only infiltration of eosinophils to the lymph nodes (33), and locus *Lmr5* carried by CcS-16, *Lmr12* and *Lmr19* control only systemic immune response to *L. major* (44, 45).

We have observed a discrepancy between genes controlling parasites in organs and genes controlling splenomegaly and hepatomegaly. In the strain CcS-9 parasite load in the liver was controlled by the loci *Lmr14*, *Lmr15,* and *Lmr27*, but only one of them, *Lmr15*, was involved in control of hepatomegaly. Similarly, in the strain CcS-16, parasite load in liver were controlled by the loci *Lmr13* and *Lmr14* (suggestive linkage), whereas hepatomegaly is determined by *Lmr14* and *Lmr15*. In the strain CcS-9 we did not detect loci controlling parasite load in spleen (see above); splenomegaly was controlled by loci *Lmr15* and *Lmr24*. In the strain CcS-16 parasite load in spleen was controlled by *Lmr11* and *Lmr13*, but none of these loci was involved in control of splenomegaly, which was determined by *Lmr14*. The difference between the genes controlling parasite load and spread to an organ and those controlling pathology of this organ reflects the multiple facets of interaction between pathogen and host.

In visceral leishmaniasis, *Leishmania* amastigotes exist and proliferate in the mononuclear phagocytic system, especially

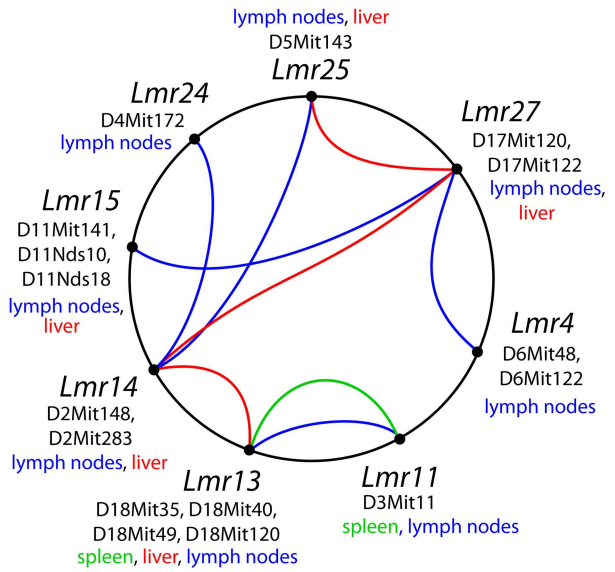

FIGURE 3 | Interactions among loci controlling parasite load after *L. major* infection. The lines that connect related loci indicate interactions controlling a certain phenotype: parasite load in lymph nodes—blue color, in spleen—green, in liver—purple. Main gene effects of the *Lmr13*, *Lmr14*, *Lmr15*, *Lmr28,* and *Lmr29* are also indicated.

spleen, liver and bone marrow. The response of immune system might lead either to parasite clearance or to unproductive inflammation resulting in organ hyperplasia (48, 49). These processes involve multiple steps that are regulated by different genes. The presented data are the first step in creating genetic model of visceralization, the most important pathology caused by *Leishmania* parasites, by identification the loci that control invasion of parasites and loci controlling the inflammatory response of the host.

Sex-Dependent Control of Parasite Load

Control of parasite load in inguinal lymph nodes is in many cases sex dependent. In the strain CcS-9, *Lmr4,* and *Lmr27* controlled parasite load in males only; *Lmr15* controlled parasite load both in females and males, but with the opposite direction: BALB/c (C) allele is linked with higher parasite load in females and lower parasite load in males. Sex has been found to influence susceptibility to many diseases, including leishmaniasis (50, 51) and genes controlling infections that are sex dependent have been observed with other infectious agents such as viruses (52, 53), bacteria (54), parasites (33, 55), fungi (56), and helminths (57). All the above mentioned loci are localized on autosomal chromosomes and thus are shared by both sexes, but the regulatory genome is sexually dimorphic (58). The regulatory genome includes steroid hormones responsive elements (59), sex-specific micro-RNA (60) and sexually dimorphic DNA methylation patterns, which vary significantly from tissue to tissue (61). Generally, the sex differences have complex functional structure and their explanation requires future molecular identification of the responsible genes.

Further studies are needed to show how these mechanisms influence frequent sex differences in human leishmaniasis. Human epidemiological studies demonstrated that women are less likely to develop leishmaniasis while men tend to be more susceptible (62), although there are exceptions (50, 63). Some epidemiological studies reported no significant sex differences in registered cases of cutaneous leishmaniasis caused by *L. tropica* (64) and *L. major* (65) between men and women. However, other studies revealed in male patients a higher incidence of cutaneous leishmaniasis caused by *L. major* and *L. tropica* (66, 67), *L. major* only (68), and also by *L. guyanensis* (69). Men were also more susceptible to visceral infection caused by *L. donovani* (70–72), *L. infantum* (73–76). As an exception to this general trend, the study in Afghanistan found that females developed more lesions and scars after *L. tropica* infection (77).

Our data do not allow conclusion of influence of sex on parasite load in organs of the strain CcS-16. We tested only females in this strain, because in previous experiments, they did not exhibit sex differences in lesion size (34), however CcS-16 might exhibit sex differences in parasite load.

CONCLUSIONS

The present study was focused on the genetic basis of one of the most important parameters of *L. major* caused leishmaniasis—parasite load in target organs. The study used a hypothesis-free experimental approach and recombinant congenic mouse strains to perform genome wide mapping of a complex system of genes that regulate dissemination of the parasite inside a mammalian organism and form a network-like structure (**Figure 3**). Host genes controlling *L. major* revealed a wide variety of heterogeneous effects that included distinct organ-specific control, single-gene effects, gene-gene interactions and sex dependent control. The presented results contribute to the understanding of genetic aspects of leishmaniasis. Mapping of these genes and subsequent identification of prospective candidate genes will allow their functional analysis. In addition, the obtained information allows making focused tests of human orthologous genes for their possible role in leishmaniasis and to elucidate pathogenesis and visceralization in individual patients.

ETHICS STATEMENT

The experiments were performed in accordance with the European Union guidelines for work with animals under the Policy of Animal Protection Law (No.246/1992), and also with the regulations of the Ministry of Agriculture of the Czech Republic (No.207/2004). The experiments 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 12/2002 and 89/2013).

AUTHOR CONTRIBUTIONS

TK and MČ conceived the study, performed experiments, interpreted the data and wrote the manuscript. VV performed experiments, analyzed the data and contributed to the writing of the manuscript. YS, HH, and MS performed the experiments. PD analyzed the data and contributed to the writing of the manuscript. ML conceived the study, interpreted data and wrote the manuscript. All authors reviewed the manuscript.

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RESEARCH ARTICLE

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A novel locus on mouse chromosome 7 that influences survival after infection with tick-borne encephalitis virus

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Abstract

Background: Tick-borne encephalitis (TBE) is the main tick-borne viral infection in Eurasia. Its manifestations range from inapparent infections and fevers with complete recovery to debilitating or fatal encephalitis. The basis of this heterogeneity is largely unknown, but part of this variation is likely due to host genetic. We have previously found that BALB/c mice exhibit intermediate susceptibility to the infection of TBE virus (TBEV), STS mice are highly resistant, whereas the recombinant congenic strain CcS-11, carrying 12.5% of the STS genome on the background of the BALB/c genome is even more susceptible than BALB/c. Importantly, mouse orthologs of human TBE controlling genes *Oas1b, Cd209*, *Tlr3, Ccr5, Ifnl3* and *Il10*, are in CcS-11 localized on segments derived from the strain BALB/c, so they are identical in BALB/c and CcS-11. As they cannot be responsible for the phenotypic difference of the two strains, we searched for the responsible STS-derived gene-locus. Of course the STS-derived genes in CcS-11 may operate through regulating or epigenetically modifying these non-polymorphic genes of BALB/c origin.

Methods: To determine the location of the STS genes responsible for susceptibility of CcS-11, we analyzed survival of TBEV-infected F_2 hybrids between BALB/c and CcS-11. CcS-11 carries STS-derived segments on eight chromosomes. These were genotyped in the F_2 hybrid mice and their linkage with survival was tested by binary trait interval mapping. We have sequenced genomes of BALB/c and STS using next generation sequencing and performed bioinformatics analysis of the chromosomal segment exhibiting linkage with TBEV survival.

Results: Linkage analysis revealed a novel suggestive survival-controlling locus on chromosome 7 linked to marker D7Nds5 (44.2 Mb). Analysis of this locus for polymorphisms between BALB/c and STS that change RNA stability and genes' functions led to detection of 9 potential candidate genes: *Cd33*, *Klk1b22*, *Siglece*, *Klk1b16*, *Fut2*, *Grwd1*, *Abcc6*, *Otog*, and *Mkrn3*. One of them, *Cd33*, carried a nonsense mutation in the STS strain.

Conclusions: The robust genetic system of recombinant congenic strains of mice enabled detection of a novel suggestive locus on chromosome 7. This locus contains 9 candidate genes, which will be focus of future studies not only in mice but also in humans.

Keywords: Tick-borne encephalitis virus (TBEV), Mouse model, Survival, Susceptibility locus, Chromosome 7, Candidate gene

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Background

Tick-borne encephalitis (TBE) is the main tick-borne viral infection in Eurasia. It is prevalent across the entire continent from Japan to France [1]. The disease is caused by tick-borne encephalitis virus (TBEV), a flavivirus of the family *Flaviviridae*, which besides TBEV includes West Nile virus (WNV), dengue virus (DENV), Zika virus (ZIKV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), and several other viruses causing extensive morbidity and mortality in humans. Ticks act as both the vector and reservoir for TBEV. The main hosts are small rodents, with humans being accidental hosts. In Europe and Russia between 5000 and 13,000 clinical cases of TBE are reported annually, with a large annual fluctuation [2]. The highest incidence of TBE is reported in western Siberia, in the Czech Republic, Estonia, Slovenia and Lithuania, but the prevalence of the disease is believed to be higher than actually reported [1, 2]. TBEV may produce a variety of clinical symptoms, from an asymptomatic disease to a fever and acute or chronic progressive encephalitis. The outcome of infection depends on the strain of virus [1], as well as on the genotype [3], sex and age of the host [4], and on the environmental and social factors [1]. Environmental and social factors influence also risk of infection.

Genetic influence on susceptibility to TBEV-induced disease has been analyzed by two main strategies: a hypothesis-independent phenotype-driven approach and a hypothesis-driven approach. Application of a genome-wide search (hypothesis-independent approach) in mouse led to identification of the gene *Oas1* (2′-5′-oligoadenylate synthetase gene) [5, 6]. A stop codon in exon 4 of the gene *Oas1b* (a natural knockout) present in majority of mouse laboratory strains causes production of protein lacking 30% of the C terminal sequence [5]. This part of molecule seems to be critical for tetramerization required for OAS1B activity leading to degradation of viral RNA. Thus, this mutation makes majority of mouse laboratory strains susceptible to flaviviruses [6, 7]. Human ortholog to this gene (*OAS1*) also modifies susceptibility to other flaviviruses (WNV) [8, 9], whereas *OAS2* and *OAS3* localized in the same cluster on chromosome 12q24.2 influence response to TBEV [3]. The polymorphic sites associated in *OAS2* and *OAS3* with susceptibility to TBEV did not resulted in amino acid changes, thus mechanisms of susceptibility control is not known [3]. The hypothesis-driven approach has focused on genes that encode molecules indicated to be involved in antiviral response by mechanistic studies [9]. These candidate genes studies revealed that polymorphisms in *CD209*/*DC-SIGN* [10], *CCR5* [11, 12], *TLR3* [12, 13], *IL10* [14] and *IFNL3*/*IL28B* [14] influence susceptibility to TBEV in humans.

Our previous study has shown that both after subcutaneous and intracerebral inoculation of European prototypic TBEV, BALB/c mice exhibited intermediate susceptibility to the infection, STS mice were highly resistant, whereas the strain CcS-11, which carries 12.5% of the STS genome on the background of the genome of the strain BALB/c [15], is even more susceptible than its two parents—BALB/c and STS [16]. Importantly, mouse orthologs of human TBEV controlling genes: *Oas1b, Cd209, Tlr3, Ccr5, Il10* and *Ifnl3* are in CcS-11 localized on segments derived from the strain BALB/c (Fig. 1), so they are identical in both BALB/c and CcS-11 and hence cannot be responsible for the phenotypic difference of the two strains. Therefore, the difference must be due to a presently unknown locus, which could be detected by a linkage study of a cross between BALB/c and CcS-11. Thus, we have generated a F_2 intercross between BALB/c and CcS-11 and performed a linkage and bioinformatics analysis. These studies revealed a novel suggestive locus on mouse chromosome 7 containing 9 potential candidate genes.

Methods

Mice

417 female F_2 offspring of an intercross between strains CcS-11 and BALB/c (mean and median age 9.5 and 9 weeks, respectively, at the time of infection) were produced at the Institute of Molecular Genetics AS CR. Mice were tested in three successive experimental groups at the Institute of Parasitology, AS CR. When used for these experiments, strain CcS-11 had undergone more than 90 generations of inbreeding. Experiments Nr. 1, 2, and 3 comprised 120, 121 and 176 F_2 mice, respectively. Sterilized pellet diet and water were supplied ad libitum. The mice were housed in plastic cages with wood-chip bedding, situated in a specific pathogen-free room with a constant temperature of 22 °C and a relative humidity of 65%.

Virus infection and disease phenotype

Experiments were performed with European prototypic TBEV strain Neudoerfl (a generous gift from Professor F. X. Heinz, Medical University of Vienna). This strain was passaged five times in brains of suckling mice before the use in this study [16]. Mice were infected subcutaneously with 10^4 pfu of the virus.

Mice were scored for mortality for a period of 35 days post-infection (p.i.) with TBEV, as well as presence of ruffled fur and paresis in three independent successive experiments at the Institute of Parasitology AS CR.

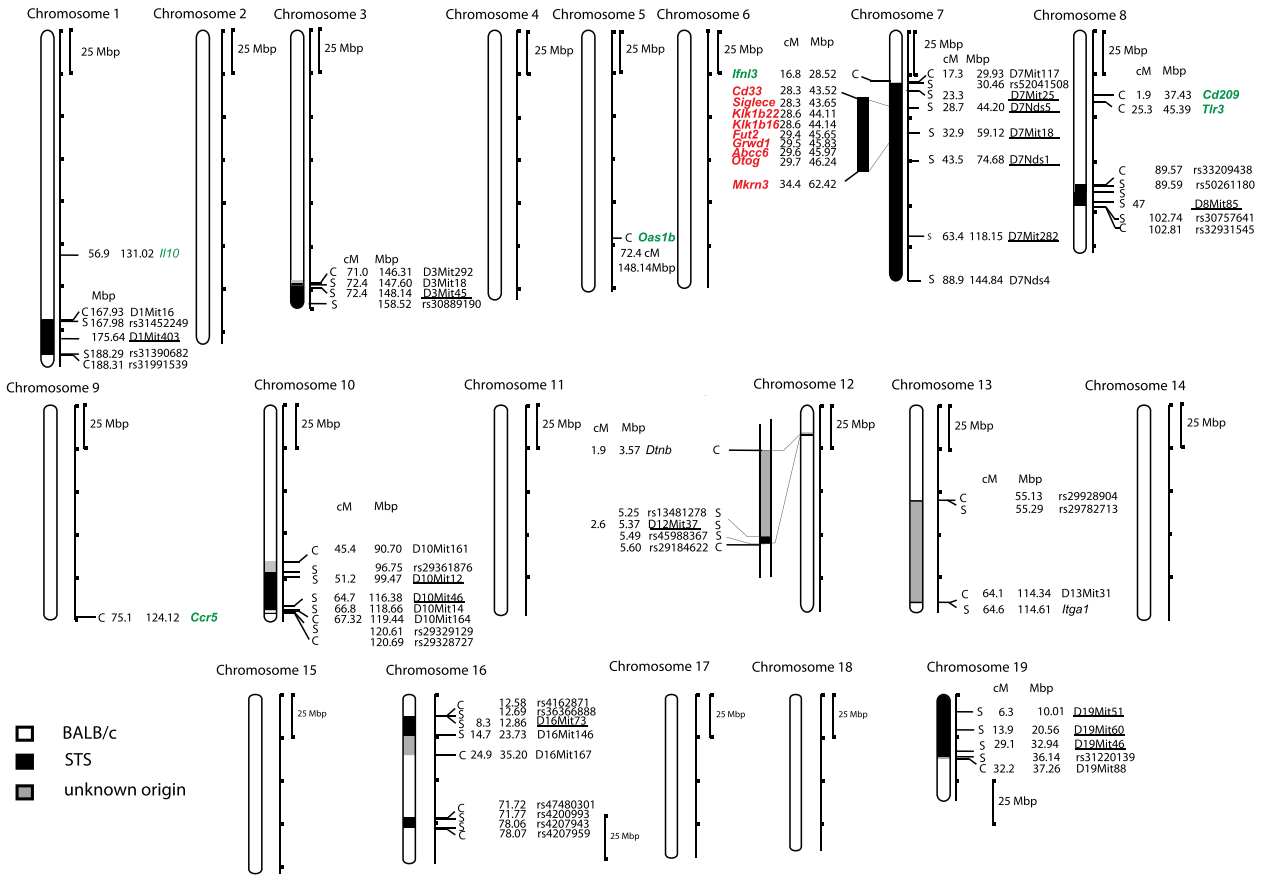

Fig. 1 Genetic composition of the strain CcS-11. The regions of STS and BALB/c origin are represented as dark and white, respectively, the boundary regions of undetermined origin are shaded. Only the markers or SNPs defining the boundaries of STS-derived segment and markers that were tested for linkage (underlined) are shown. Genes *Oas1b*, *Cd209*, *Tlr3*, *Ccr5*, *Ifnl3* and *Il10*, known to control susceptibility to TBEV are shown in green, potential candidate genes *Cd33*, *Klk1b22*, *Siglece*, *Klk1b16*, *Fut2*, *Grwd1*, *Abcc6*, *Otog*, and *Mkrn3* detected in current study are shown in red

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 [17]. These differential regions were genotyped in the F_2 hybrid mice between CcS-11 and BALB/c using 16 microsatellite markers (Generi Biotech, Hradec Králové, Czech Republic): D1Mit403, D3Mit45, D7Mit25, D7Nds5, D7Mit18, D7Nds1, D7Mit282, D7Mit259, D8Mit85, D10 Mit12, D10Mit46, D12Mit37, D16Mit73, D19Mit51, D19Mit60, D19Mit46 (Fig. 1) as described in [17].

Statistical analysis

Survival, ruffled fur and paresis were treated as binary phenotypes (death/survival; presence/absence of symptom), and binary trait interval mapping was performed [18, 19]. A permutation test [20] was used to assess significance. This takes account of the limited genetic difference between the strains BALB/c and CcS-11. On the basis of 10,000 permutation replicates, the 5% significance LOD threshold was 2.56; the 10% threshold was 2.23. The Pearson correlation coefficient between presence of death and paresis was computed by the program Statistica for Windows 12.0 (StatSoft, Inc., Tulsa, OK).

Detection of polymorphisms that change RNA stability and genes' functions

We have sequenced the genomes of strains BALB/c and STS using next generation sequencing (NGS) system HiSeq 2500 (Illumina) (12× coverage). NGS data was preprocessed using software Trimmomatic [21] and overlapped paired reads were joined by software Flash [22]. Alignment—reference mouse sequence mm10 (build GRCm38)—was performed using BWA (Burrows-Wheeler Aligner) [23] program. Mapped reads were sorted and indexed, duplicated reads were marked. Segment covering peak of linkage on chromosome 7 from 36.2 to 74.5 Mb was inspected for polymorphisms between BALB/c and STS that change RNA stability and

genes' functions. Local realignment around indels, base recalibration and variants filtration were performed using software GATK (The Genome Analysis Toolkit) [24]. Variant annotation and effect prediction was performed by software SnpEff [25]. IGV (Integrated Genome Viewer) was used for visualization of results [26].

Results

Binary trait linkage analysis revealed a suggestive locus on chromosome 7 near D7Nds5 affecting the binary trait (death/survival) (LOD = 2.15), with a corresponding genome-scan-adjusted *P* value = 0.12 (Fig. 2a). The 1-LOD support interval spans from D7Mit25 to D7Nds1. The STS allele both in homozygotes and heterozygotes was associated with a higher death rate in each of the three separate experimental groups (Fig. 2b), and in the pooled data (Fig. 3a), so its presence in CcS-11 enhances even more the overall susceptibility determined by the BALB/c background. Ruffled fur was observed in only 8% of mice, so it was not suitable for statistical analysis. Paresis was less frequent than mortality (n = 60 vs. 102) and not all paretic mice died, but the two phenotypes were positively correlated (Pearson correlation 0.53). Moreover, frequency of paresis in the three D7Nds5 genotypes (Fig. 3b), although not significantly different, was biologically compatible with the survival data, because D7Nds5 CC homozygotes had the highest survival rate, and the lowest percentage of paresis.

We have sequenced genomes of BALB/c and STS and analyzed the segment covering peak of linkage on chromosome 7 from 36.2 to 74.5 Mb for polymorphisms between BALB/c and STS that change RNA stability and genes' functions. This revealed 9 potential candidate genes: *Cd33* (CD33 antigen), *Klk1b22* (kallikrein 1-related peptidase b22), *Siglece* (sialic acid binding Ig-like lectin E), *Klk1b16* (kallikrein 1-related peptidase b16), *Fut2* (fucosyltransferase 2), *Grwd1* (glutamate-rich WD repeat containing 1), *Abcc6* (ATP-binding cassette,

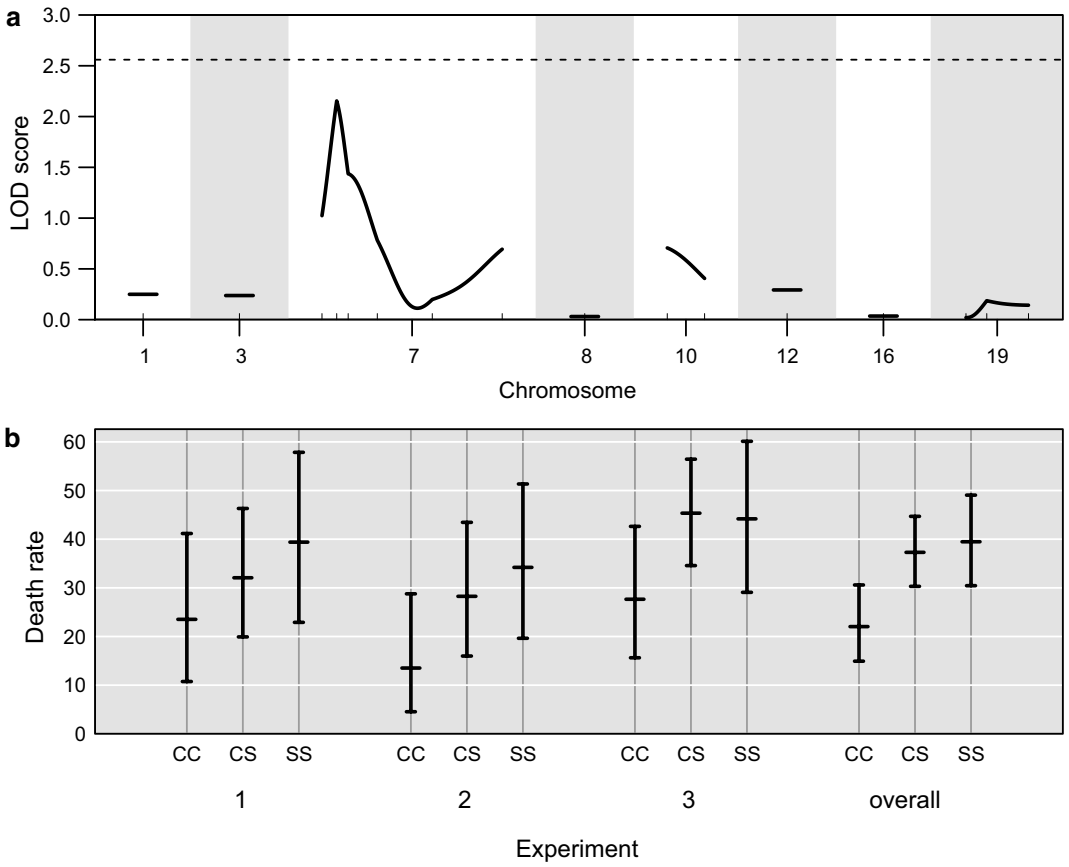

Fig. 2 Genetic influence on susceptibility to TBEV in an F_2 intercross between BALB/c and CcS-11. **a** LOD curves from binary trait interval mapping for death/survival. A dashed horizontal line is plotted at the 5% significance threshold, adjusting for the genome scan. **b** A plot of the death rate as a function of genotype at marker D7Nds5 and experiment, with 95% confidence intervals. C and S indicate the presence of BALB/c and STS allele, respectively. The S allele is associated with a higher death rate. The numbers of mice in the experiments 1, 2, and 3 were 120, 121 and 176, respectively

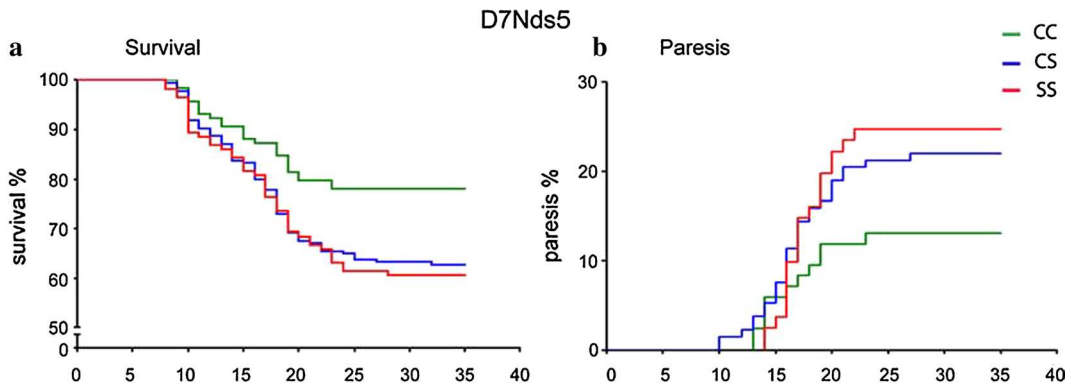

Fig. 3 Differential survival (**a**) and incidence of paresis (**b**) of and in F_2 hybrid mice carrying CC, CS and SS genotype at the marker D7Nds5 (n.s) after TBEV infection. Mice were infected subcutaneously with 10^4 pfu of the TBEV strain Neudoerfl and observed for lethality for 35 days

sub-family C (CFTR/MRP), member 6), *Otog* (otogelin), and *Mkrn3* (makorin, ring finger protein, 3) (Table 1, Fig. 1).

One of these genes, *Cd33*, carried in the strain STS a nonsense mutation. Other changes in the strain STS in comparison with BALB/c (and the reference strain C57BL/6) represented single amino acids change in Siglec E, KLK1B22, KLK1B16, FUT2 and OTOG. The BALB/c strain had in comparison with STS (and the reference strain C57BL/6) deletion of two amino acids in GRWD1, insertion of four amino acids in MKRN3 and single amino acid change in and ABCC6 (Table 1).

Discussion

CD33 and Siglec E belong to family of CD33-related sialic-acid-binding immunoglobulin-like lectins (CD33r-Siglecs). They are ITIM-containing inhibitory receptors, which are involved in regulation of inflammatory and immune responses [27]. Gene *Cd33* carried in the strain STS a nonsense mutation (Table 1). Product of this gene is in mouse expressed on myeloid precursors and cells of myeloid origin [28] and on microglial cells [29]. It can inhibit response to amyloid plaques and its deletion leads to protection in the mouse model of Alzheimer disease (AD) [29] and in humans some *CD33* genetic variants are associated with late-onset AD [30]; its potential role in pathology of TBEV might be associated with its regulatory role in inflammatory responses. Gene *Siglece* carried in the strain STS a single amino acid change. Siglec E is expressed on microglia and inhibits neurotoxicity triggered by neural debris [31], which might have protective role against damage induced by flaviviruses.

A single amino acid change was present in KLK1B22 and KLK1B16. Kallikreins are serine proteases that might both help to fight infection by activating complement system [32], as well as aggravate disease symptoms by releasing bradykinin, which causes alterations in vascular permeability [33]. Their role in defense against flaviviruses has not been described. Kallikrein-bradykinin system have been described to contribute to protection against *Leishmania* [34] and *Trypanosoma cruzi* [35] parasites in mice. Interestingly, on the mouse chromosome 7 were in the strain CcS-11 mapped loci *Lmr21* and *Tbbr3* that control susceptibility to *L. major* [36] and *T. b. brucei* [17], respectively. However, both loci are mapped on a long chromosomal segment, thus other gene(s) might be responsible for their effect.

FUT2 have been described to influence control of a wide range of pathogens such as noroviruses [37], rotaviruses [38], HIV [39], and *Escherichia coli* [40] in humans, and to *Helicobacter pylori* in mouse [41], but its role in resistance to flaviviruses is not known.

Makorin 1 induces degradation of WNV capsid which might protect host cells [42]. The E3 ligase domain responsible for MKRN1 effect is present also in MKRN3 [43]. Thus, gene *Mkrn3* might have relationship to defense against flaviviruses. Similarly, possible role of *Otog, Grwd1* and *Abcc6* in resistance to TBEV remains to be elucidated.

Public database BioGPS shows that all the candidate genes are in uninfected mice expressed in tissues such as brain, spleen and liver (Table 2). Brain is the main target for the virus; however, during the extraneural phase of the infection, several tissues and organs in the body are infected, including spleen and liver [44]. Highest expression in these tissues exhibits *Cd33* and *Siglece* with expression in microglia ten times higher than median value (> 10M), *Cd33* and *Klk1b22* are highly expressed in spleen (> 3M), > 10M expression of these two genes is also observed in bone marrow; *Siglece* is also highly expressed in bone (> 3M) and bone marrow macrophages (> 3M), whereas *Cd33* is highly expressed

Table 1 List of candidate genes in TBEV susceptibility locus

Position Bp	Reference genotype C57BL/6	Genotype BALB/c	Genotype STS	Protein position of amino acid	Reference amino acid	Alteration	Type of change	Gene symbol	Transcription status	Gene name	Gene ID: MGI	Gene ID: NCBI
43,528,893	C/C	C/C	T/T	353	G	K	Single AA Change	*Cd33*	KNOWN	CD33 antigen	99,440	12,489
43,532,167	G/G	G/G	A/A	190	R	*	Nonsense Mutation	*Cd33*	KNOWN	CD33 antigen	99,440	12,489
43,659,827	G/G	G/G	T/T	102	D	E	Single AA Change	*Siglece*	KNOWN	Sialic acid binding Ig-like lectin E	1,932,475	83,382
44,115,970	A/A	A/A	C/A	115	L	Y	Single AA Change	*Klk1b22*	KNOWN	Kallikrein 1-related peptidase b22	95,291	13,646
44,140,534	G/G	G/G	C/C	76	G	A	Single AA Change	*Klk1b16*	KNOWN	Kallikrein 1-related peptidase b16	891,982	16,615
45,650,779	G/G	G/G	A/A	190	R	W	Single AA Change	*Fut2*	KNOWN	Fucosyltransferase 2	109,374	14,344
45,830,054	CTCTTCA/CTCTTCA	C/C	CTCTTCA/CTCTTCA	129	ED	.	Deletion	*Grwd1*	KNOWN	Glutamate-rich WD repeat containing 1	2,141,989	101,612
45,977,290	C/C	A/A	C/C	1448	V	L	Single AA Change	*Abcc6*	KNOWN	ATP-binding cassette, sub-family C (CFTR/MRP), member 6	1,351,634	27,421
46,262,804	C/C	C/C	T/T	748	R	W	Single AA Change	*Otog*	KNOWN	Otogelin	1,202,064	18,419
62,419,214	C/C	CGGCATTGGCACT/CGGCATTGGCACT	C/C	275	P	PVPMP	Insertion	*Mkrn3*	KNOWN	Makorin, ring finger protein, 3	2,181,178	22,652

Table shows differences between BALB/c and STS in DNA and protein sequences in potential candidate genes. Table shows also sequences of the reference mouse strain C57BL/6

Table 2 Expression of potential candidate genes in organs and cells of uninfected mice

Gene symbol	*Cd33*		*Siglece*		*Klk1b22*		*Klk1b16*		*Fut2*		*Grwd1*		*Abcc6*		*Otog*		*Mkrn3*	
Gene ID: MGI	99,440		1,932,475		95,291		891,982		109,374		2,141,989		1,351,634		1,202,064		2,181,178	
Gene ID: NCBI	12,489		83,382		13,646		16,615		14,344		101,612		27,421		18,419		22,652	
Median	5.2		4.7		4.6		4.6		4.9		163		4.6		4.95		4.8	
Organs																		
Bone	77.05	> 10M	20.82	> 3M	4.64	~ M	4.64	~ M	4.86	~ M	86.89	<M	4.64	~ M	4.95	~ M	4.64	~ M
Bone marrow	114.05	> 10M	51.63	> 10M	4.64	~ M	4.64	~ M	4.88	~ M	106.44	~ M	4.64	~ M	4.79	~ M	4.66	~ M
Brain																		
Amygdala	4.97	~ M	4.67	~ M	4.64	~ M	4.64	~ M	4.86	~ M	73.20	<M	4.64	~ M	4.95	~ M	4.81	~ M
Cerebellum	4.91	~ M	4.76	~ M	5.24	~ M	5.64	~ M	5.39	~ M	26.75	<M	4.64	~ M	4.94	~ M	5.86	~ M
Cerebral cortex	4.91	~ M	4.67	~ M	4.64	~ M	4.64	~ M	4.90	~ M	93.30	<M	4.64	~ M	4.94	~ M	4.64	~ M
Hippocampus	4.91	~ M	4.67	~ M	4.64	~ M	4.64	~ M	4.86	~ M	51.91	<M	4.64	~ M	4.95	~ M	4.76	~ M
Olfactory bulb	4.89	~ M	4.67	~ M	4.64	~ M	4.67	~ M	4.86	~ M	38.09	<M	4.64	~ M	4.95	~ M	19.91	> 3M
Eye																		
Eyecup	13.71	~ 3M	4.67	~ M	6.64	> M	4.64	~ M	5.80	~ M	180.25	~ M	4.64	~ M	4.95	~ M	7.49	> M
Lens	9.26	> M	6.18	~ M	4.64	~ M	7.54	> M	5.80	~ M	392.8	> M	56.53	~ 10M	4.95	~ M	13.36	> M
Retina	5.72	~ M	4.67	~ M	4.74	~ M	4.64	~ M	4.86	~ M	147.73	<M	4.64	~ M	4.95	~ M	38.78	~ 10M
Intestine large	4.86	<M	4.67	~ M	486.82	≫30M	6.13	> M	91.36	> 3M	62.6	<M	4.64	~ M	4.94	~ M	4.64	~ M
Intestine small	4.91	~ M	4.68	~ M	35.44	> 3M	4.66	~ M	6.45	~ M	128.54	~ M	5.80	> M	4.95	~ M	4.64	~ M
Kidney	4.87	~ M	4.67	~ M	607.67	> 30M	15.57	~ 3M	10.44	> M	80.87	<M	4.64	~ M	4.95	~ M	4.64	~ M
Lacrimal gland	4.92	~ M	4.67	~ M	541.25	> 30M	187.21	> 30M	15.28	> M	286.48	> 3M	4.64	~ M	4.95	~ M	4.64	~ M
Liver	4.89	~ M	5.09	~ M	4.64	~ M	6.14	> M	4.86	~ M	161.71	~ M	920.68	≫30M	4.95	~ M	4.64	~ M
Lymph nodes	85.8	> 3M	4.68	~ M	4.64	~ M	4.64	~ M	4.99	~ M	97.85	<M	4.64	~ M	4.95	~ M	4.64	~ M
Pancreas	7.40	~ M	5.15	~ M	1806.5	> 30M	22.47	> 3M	10.44	> M	25.91	~ M	4.64	~ M	9.28	> M	4.64	~ M
Pituitary	4.89	~ M	4.67	~ M	13.10	~ 3M	114.09	~ 30M	7.07	> M	61.18	<M	4.64	~ M	4.95	~ M	4.64	~ M
Prostate	5.25	~ M	4.75	~ M	4.64	~ M	4.66	~ M	42.15	> 3M	48.62	<M	4.64	~ M	4.95	~ M	4.64	~ M
Salivary gland	5.62	~ M	4.67	~ M	36,542.06	> 1000M	26,974.63	> 1000M	5.95	~ M	189.83	~ M	4.64	~ M	4.99	~ M	5.25	~ M
Spleen	15.39	~ 3M	9.02	> M	13.11	~ 3M	4.64	~ M	4.91	~ M	122.09	<M	4.64	~ M	4.95	~ M	4.81	~ M
Stomach	4.89	~ M	4.65	~ M	81.39	> 10M	4.64	~ M	117.61	> 10M	73.50	<M	4.64	~ M	4.95	~ M	4.64	~ M
Testis	5.40	~ M	4.67	~ M	567.00	> 30M	30.89	> 3M	16.28	~ 3M	86.24	<M	10.55	> M	4.95	~ M	6.04	~ M
Uterus	4.91	~ M	4.67	~ M	4.64	~ M	4.64	~ M	222.61	> 30M	131.6	<M	4.64	~ M	4.95	~ M	5.84	~ M
Cells																		
B cells_marginal_zone	25.26	> 3M	4.67	~ M	4.97	~ M	4.64	~ M	4.86	~ M	446.31	~ 3M	4.64	~ M	4.95	~ M	4.64	~ M
Common myeloid progenitor	11.3	> M	6.18	~ M	5.77	~ M	5.05	~ M	5.20	~ M	1192.34	> 3M	4.64	~ M	5.91	~ M	4.64	~ M
Dendritic lymphoid cells	9.83	> M	4.67	~ M	4.64	~ M	4.64	~ M	4.86	~ M	369.07	> M	4.64	~ M	5.11	~ M	4.64	~ M

Table 2 (continued)

Gene symbol	*Cd33*		*Siglece*		*Klk1b22*		*Klk1b16*		*Fut2*		*Grwd1*		*Abcc6*		*Otog*		*Mkrn3*	
Dendritic cells myeloid CD8a−	77.51	> 10M	4.96	~ M	4.64	~ M	4.64	~ M	4.86	~ M	299.19	> M	4.64	~ M	4.95	~ M	4.64	~ M
Dendritic plasmacytoid B220+	140.59	~ 30M	4.74	~ M	88.52	> 10M	4.64	~ M	4.86	~ M	238.95	> M	4.64	~ M	5.14	~ M	4.64	~ M
Granul ocytes mac1 + gr1+	651.27	> 30M	6.87	> M	4.64	~ M	4.64	~ M	5.04	~ M	53.79	<M	4.64	~ M	4.95	~ M	4.64	~ M
Hematopoietic stem cells	42.14	> 3M	4.8	~ M	4.96	~ M	4.64	~ M	6.41	> M	1445.80	~ 10M	4.64	~ M	4.95	~ M	5.19	~ M
Macrophage_bone_marrow	11.64	> M	14.68	> 3M	4.64	~ M	4.64	~ M	4.86	~ M	308.83	> M	4.64	~ M	4.95	~ M	4.64	~ M
Mast cells	214.54	> M	4.67	~ M	4.64	~ M	4.64	~ M	4.83	~ M	55.07	<M	4.64	~ M	4.95	~ M	4.64	~ M
Mega_erythrocyte progenitor	5.31	~ M	4.67	~ M	7.57	> M	4.64	~ M	6.47	> M	2842.61	> 10M	4.64	~ M	8.07	> M	4.64	~ M
Microglia	99.81	> 10M	79.64	> 10M	4.64	~ M	4.64	~ M	4.86	~ M	269.08	> M	4.64	~ M	4.95	~ M	4.64	~ M
Osteoclasts	363.46	> 30M	4.77	~ M	7.67	> M	4.64	~ M	4.99	~ M	327.07	> M	4.64	~ M	5.31	~ M	4.64	~ M
T-cellsCD4+	4.91	~ M	5.00	~ M	7.57	> M	4.82	~ M	4.86	~ M	621.74	> 3M	4.64	~ M	5.06	~ M	4.64	~ M
T-cellsCD8+	4.91	~ M	4.67	~ M	13.55	~ 3M	4.64	~ M	5.29	~ M	715.19	> 3M	4.64	~ M	6.13	> M	4.64	~ M
T-cells FoxP3+	25.67	> 3M	4.67	~ M	4.64	~ M	4.96	~ M	4.93	~ M	584.63	~ 3M	4.64	~ M	4.95	~ M	4.64	~ M

Data were compiled from public database BioGps (http://biogps.org) May 6, 2018. First column: relative units; second column: relationship to median (M); M = median value across all samples for a single probe set

in granulocytes (> 30M), plasmacytoid dendritic cells (> 30M), osteoclasts (> 30M), myeloid dendritic cells (> 10M), in spleen (> 3M), lymph nodes (> 3M), eyecup (> 3M), B cells in marginal zone (> 3M) and in $FoxP^+$ T cells (> 3M). For both *Klk1b22* and *Klk1b16* is characteristic very high expression in salivary gland (> 1000M) and high expression in lacrimal gland (> 30M). *Klk1b22* is also highly expressed in large intestine (> 30M), kidney (> 30M), pancreas (> 30M), testis (> 30M), stomach (> 10M), plasmacytoid dendritic cells (> 10M), small intestine (> 3M), spleen (> 3M) and CD8 + T cells (> 3M), whereas *Klk1b16* is also highly expressed in pituitary (> 30M), kidney (> 3M), pancreas (> 3M) and testis (> 30M). Highest expression of *Fut2* was observed in uterus (> 30M), and in stomach (> 10M), it was also highly expressed in large intestine (> 3M), prostate (> 3M) and in testis (> 3M). GRWD1 was described to play a role in ribosome biogenesis and during myeloid differentiation [45]. High expression level in hematopoietic stem cells (> 10M), mega erythrocyte progenitors (> 10M), granulocytes (> 10M), common myeloid progenitor (> 3M) supports this finding, but it is also expressed in several T cell subpopulations (> 3M), B cells in marginal zone (> 3M), as well as in lacrimal gland. *Abcc6* is highly expressed in liver (≫ 30M) and in lens (> 10M) and *Mrkn3* is highly expressed in retina (10M) and in olfactory bulb (> 3M). The expression data further support a potential role of detected candidate genes in defense against TBEV, but they must be in the future complemented with data describing gene expression after TBEV infection.

We have found a susceptibility allele of a locus on chromosome 7 in the resistant strain STS. This apparent paradox is likely caused by the fact that most inbred mouse strains were produced without an intentional selective breeding for a specific quantitative phenotype (like susceptibility to specific infections). Therefore they inherited randomly from their non-inbred ancestors susceptible alleles at some loci and resistant alleles at others, so that their overall susceptibility phenotype depends on the relative number of both types of alleles. Such finding is not unique, as susceptibility alleles originating from resistant strains were found in susceptibility studies of other infectious diseases [17, 46, 47] and colon cancer [48]. Similarly, in different in vitro tests of immune responses a low-responder allele was identified in a high responding strain [49] or vice versa [50]. Another explanation might be presence of BALB/c allele interacting with STS allele on chromosome 7. Demonstration of such interaction would require further experiments. We have already observed interaction of STS and BALB/c alleles leading to extreme phenotypes in susceptibility to *L. major* [51] and *L. tropica* [47].

Conclusion

Mapping of TBEV controlling genes in mice is not easy due to presence of a strong TBEV controlling gene *Oas1b,* which is identical both in BALB/c and CcS-11, as well as in majority of laboratory mouse strains [6, 7] and masks effects of other controlling genes. Therefore using a powerful genetic system—recombinant congenic strains, we succeeded in mapping novel TBEV susceptibility locus on chromosome 7 and identified 9 potential candidate genes. Products of some of these genes have been described to participate in defense against flaviviruses, the role of the others is unknown. The genes detected here will be focus of future studies that will include characterization of candidate gene(s) products in BALB/c and CcS-11, introducing modification to candidate genes and study their influence on disease outcome in mouse, and study influence of polymorphisms in human orthologs of candidate genes on susceptibility to TBEV in humans.

List of abbreviations

Abcc6 (ATP-binding cassette, sub-family C (CFTR/MRP), member 6), mouse gene; *ABCC6*-human gene; ABCC6-protein (gene product); *Ccr5:* chemokine (C–C motif) receptor 5*; Cd209:* CD209 antigen; *Cd33* (CD33 antigen); *Fut2* (fucosyltransferase 2); *Grwd1* (glutamate-rich WD repeat containing 1); *Ifnl3:* interferon lambda 3 (synonym *Il28b*); *Il10*: interleukin 10; *Klk1b16* (kallikrein 1-related peptidase b16); *Klk1b22* (kallikrein 1-related peptidase b22); *Mkrn3* (makorin, ring finger protein, 3); *Oas1b:* 2′-5′-oligoadenylate synthetase gene; *Otog* (otogelin); pfu: Plaque-forming unit; RC: Recombinant congenic; s.c: Subcutaneous; *Siglece* (sialic acid binding Ig-like lectin E); TBE: Tick-borne encephalitis; TBEV: Tick-borne encephalitis virus; *Tlr3:* toll-like receptor 3.

According to current gene and protein nomenclature, mouse gene symbols are italicized, with only the first letter in upper-case (e.g. *Cd33*). Protein symbols are not italicized, and all letters are in upper-case (e.g. CD33). Human gene symbols are in upper-case and are italicized (e.g. *CD33*). Protein symbols are identical to their corresponding gene symbols except that they are not italicized (e.g. CD33).

Authors' contributions

ML conceived and designed the research, analyzed the experiments, coordinated collaborations; and wrote the manuscript. MP, YS, MŠ, DR, VV, JV, LM, JS and MS performed the experiments and participated in the design of experiments and analysis of data. HS performed sequence analysis. KWB performed statistical analysis. HS and JV analyzed bioinformatics data. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The research had complied with all relevant European Union guidelines for work with animals and was in accordance with the Czech national law and guidelines on the use of experimental animals and protection of animals against cruelty (the Animal Welfare Act Number 246/1992 Coll.). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Institute of Parasitology and of the Departmental Expert Committee for the Approval of Projects of Experiments on Animals of the Academy of Sciences of the Czech Republic (Permit Number: 165/2010).

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