Genetic Control of Resistance to \textit{Trypanosoma brucei brucei} Infection in Mice

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\section*{Abstract}

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

\textbf{Methods}: We studied the genetic control of survival after \textit{T. b. brucei} infection using recombinant congenic (RC) strains, which have a high mapping power. Each RC strain of BALB/c-c-STS/A (CsS/Dem) series contains a different random subset of 12.5\% genes from the parental “donor” strain STS/A and 87.5\% genes from the “background” strain BALB/c. Although BALB/c and STS/A mice are similarly susceptible to \textit{T. b. brucei}, the RC strain CsS-11 is more susceptible than either of them. We analyzed genetics of survival in \textit{T. b. brucei}-infected \textit{F}_2 hybrids between BALB/c and CsS-11. CsS-11 strain carries STS-derived segments on eight chromosomes. They were genotyped in the \textit{F}_2 hybrid mice and their linkage with survival was tested by analysis of variance.

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

\textbf{Conclusion}: This study presents the first identification of chromosomal loci controlling susceptibility to \textit{T. b. brucei} infection. While mapping in \textit{F}_2 hybrids of inbred strains usually has a precision of 40–80 Mb, in RC strains we mapped \textit{Tbbr2} to a 2.15 Mb segment containing only 26 genes, which enable an effective search for the candidate gene. Definition of susceptibility genes will improve the understanding of pathways and genetic diversity underlying the disease and may result in new strategies to overcome the active subversion of the immune system by \textit{T. b. brucei}.

\section*{Introduction}

Sleeping sickness (African trypanosomiasis) continues to pose a major threat to 60 million people in 36 countries in sub-Saharan Africa. The estimated number of new cases is currently between 50 000 and 70 000 per year (WHO 2006 – African trypanosomiasis - http://www.who.int/mediacentre/factsheets/fs259/en/). The disease is caused by infection with the tsetse fly-transmitted \cite{foot1} protozoan haemoflagellate \textit{Trypanosoma brucei}, which has three major sub-species: \textit{T. b. gambiense}, \textit{T. b. rhodesiense} and \textit{T. b. brucei}. Two of them, \textit{T. b. gambiense} and \textit{T. b. rhodesiense} cause sleeping sickness in humans and can also infect animals; thus domestic and wild animals are an important parasite reservoir (WHO 2006 - http://www.who.int/mediacentre/factsheets/fs259/en/). The third species, \textit{T. b. brucei} infects a wide range of mammals, but is unable to infect humans because it lacks the SRA (serum resistance-associated) protein that prevents lysis induced by Apolipoprotein L1, which is present in normal human serum \cite{foot2,foot3}. \textit{T. b. equiperdum} and \textit{T. b. evansi}, which are derived from \textit{T. b. brucei}, are adapted to transmission without development in tsetse fly, allowing these parasites to spread outside the African tsetse belt \cite{foot4}.

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

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Trypanosoma brucei are extracellular protozoa transmitted to mammalian host by the tsetse fly. They developed several mechanisms that subvert host’s immune defenses. Therefore analysis of genes affecting host’s resistance to infection can reveal critical aspects of host-parasite interactions. Trypanosoma brucei brucei infects many animal species including livestock, with particularly severe effects in horses and dogs. Mouse strains differ greatly in susceptibility to T. b. brucei. However, genes controlling susceptibility to this parasite have not been mapped. We analyzed the genetic control of survival after T. b. brucei infection using CcS/Dem recombinant congenic (RC) strains, each of which contains a different random set of 12.5% genes of their donor parental strain STS/A on the BALB/c genetic background. The RC strain CcS-11 is even more susceptible to parasites than BALB/c or STS/A. In F2 hybrids between BALB/c and CcS-11 we detected and mapped four loci, Tbrb1-4 (Trypanosoma brucei brucei response 1-4), that control survival after T. b. brucei infection. Tbrb1 (chromosome 3) and Tbrb2 (chromosome 12) have independent effects; Tbrb3 (chromosome 7) and Tbrb4 (chromosome 19) were detected by their mutual inter-genic interaction. Tbrb2 was precision mapped to a segment of 2.15 Mb that contains 26 genes.

Author Summary

Trypanosoma brucei are extracellular protozoa transmitted to mammalian host by the tsetse fly. They developed several mechanisms that subvert host’s immune defenses. Therefore analysis of genes affecting host’s resistance to infection can reveal critical aspects of host-parasite interactions. Trypanosoma brucei brucei infects many animal species including livestock, with particularly severe effects in horses and dogs. Mouse strains differ greatly in susceptibility to T. b. brucei. However, genes controlling susceptibility to this parasite have not been mapped. We analyzed the genetic control of survival after T. b. brucei infection using CcS/Dem recombinant congenic (RC) strains, each of which contains a different random set of 12.5% genes of their donor parental strain STS/A on the BALB/c genetic background. The RC strain CcS-11 is even more susceptible to parasites than BALB/c or STS/A. In F2 hybrids between BALB/c and CcS-11 we detected and mapped four loci, Tbrb1-4 (Trypanosoma brucei brucei response 1-4), that control survival after T. b. brucei infection. Tbrb1 (chromosome 3) and Tbrb2 (chromosome 12) have independent effects; Tbrb3 (chromosome 7) and Tbrb4 (chromosome 19) were detected by their mutual inter-genic interaction. Tbrb2 was precision mapped to a segment of 2.15 Mb that contains 26 genes.

Materials and Methods

Mice
Mice of strains tested for survival BALB/cHeA (BALB/c) (10 females, 10 males), STS/A (10 females, 10 males), CcS-5 (10 females, 10 males), CcS-11 (10 females, 10 males), CcS-16 (9 females, 9 males) and CcS-20 (10 females, 10 males) were 13 to 23 weeks old (mean 17, median 17) at the time of infection. Splenomegaly, hepatomegaly, body weight changes and serum levels of seven cytokines and chemokines were analyzed using females of BALB/c (22 infected, 22 non-infected), STS (17 infected, 13 non-infected) and CcS-11 (25 infected, 26 non-infected), which were 8 to 19 week old (mean 13, median 13) at the time of infection. When used for these experiments, CcS/Dem strains passed more than 38 generation of inbreeding and therefore were highly homozygous. The regions of RCS’ genomes inherited from the BALB/c or STS parents were defined [32]. 169 F2 hybrids between CcS-11 and BALB/c (age 22 and 23 weeks at the time of infection) were produced at the Institute of Molecular Genetics. They comprised 85 females and 84 males and were tested simultaneously as a single experimental group. During the experiment, mice were placed into individually ventilated cages behind a barrier. The research had complied with all relevant European Union guidelines for work with animals and was approved by the Institutional Animal Care Committee of the Institute of Molecular Genetics AS CR and by Departmental Expert Committee for the Approval of Projects of Experiments on Animals of the Academy of Sciences of the Czech Republic.

Parasites
The strain of Trypanosoma brucei brucei (AnTar1) was a generous gift of Jan van den Abbeele, Institute of Tropical Medicine “Prince Leopold”, Antwerp, Belgium. Parasites stored in liquid nitrogen were thawed and used to infect BALB/c males by intra-peritoneal...
inoculation. Four to five days after infection, 10 μl of tail blood was collected, diluted in 90 μl of 1% formaldehyde in PBS, and the trypanosomes were counted in a Bürker counting chamber. Subsequently, tail blood was diluted in RPMI containing L-glutamine, sodium bicarbonate and glucose (Cat. Nr. R8758, Sigma, St. Louis, MO) in order to contain appropriate numbers of parasites for inoculation (Please see below).

Trypanosomiasis challenge

Mice were inoculated intraperitoneally with 2.5 × 10^4 blood-stream forms of T. b. brucei (AnTat1 strain) in 50 μl of RPMI containing L-glutamine, sodium bicarbonate and glucose (Cat. Nr. R8758, Sigma, St. Louis, MO). Survival time was measured in days following the day of challenge (day 0).

Disease phenotype

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

Cytokine and chemokine levels

Levels of GM-CSF (granulocyte-macrophage colony-stimulating factor), CCL2 (chemokine (C-C motif) ligand 2/MCP-1 (monocyte chemotactic protein-1), CCL3/MIP-1α (macrophage inflammatory protein-1α), CCL4/MIP-1β (macrophage inflammatory protein-1β), CCL5/RANTES (regulated upon activation, normal T-cell expressed, and secreted), CCL7/MCP-3 (monocyte chemotactic protein-3) and TNF-α, in serum were determined using Mouse chemokine 6-plex kit (Bender MedSystems, Vienna, Austria) and Mouse TNF-α simplex kit as multiplex assay. The kit contains two sets of beads of different size internally dyed with different intensities of fluorescent dye. The set of small beads is used for GM-CSF, CCL5/RANTES, CCL4/MIP-1β and TNF-α and set of large beads for CCL3/MIP-1α, CCL2/MCP-1 and CCL7/MCP-3. The beads are coated with antibodies specifically reacting with each of the analytes (chemokines) to be detected in the multiplex system. A biotin secondary antibody mixture binds to the analytes captured by the first antibody. Streptavidin–Phycocyanin binds to the biotin conjugate and emits fluorescent signal. The experiment procedure was performed in the 96 well filter plates (Millipore, Billerica, MA, USA) according to the protocol of Bender MedSystem. Beads were analyzed on flow cytometer LSR II (BD Biosciences, San Jose, CA, USA). Concentrations of cytokines were determined by Flow Cytomix Pro 2.4 software. The limit of detection of each analyte was determined to be for GM-CSF 12.2 pg/ml, CCL2/MCP-1 42 pg/ml, CCL7/MCP-3 1.4 pg/ml, CCL3/MIP-1α 1.8 pg/ml, CCL4/MIP-1β 14.9 pg/ml, CCL5/RANTES 6.1 pg/ml, TNF-α 2.1 pg/ml respectively.

Genotyping of F2 mice

DNA was isolated from tails using a standard proteinase procedure. The strain CsC-11 differs from BALB/c at STS-derived regions on eight chromosomes [32]. These differential regions were typed in the F2 hybrid mice between CsC-11 and BALB/c using 14 microsatellite markers [Research Genetics, Huntsville, AL, and Generi Biotech, Hradec Králové, Czech Republic]: D1Mit103, D3Mit45, D7Mit25, D7Mit18, D7Mit282, D7Mit259, D8Mit85, D10Mit46, D10Mit12, D12Mit37, D16Mit73, D19Mit51, D19Mit60 and D19Mit46 (Table S1). The average distance between any two markers in the chromosomal segments derived from the strain STS or from the nearest BALB/c derived markers was 8.7 cM. DNA was amplified in a 20-μl PCR reaction with 0.11 μM of forward and reverse primer, 0.2 mM concentration of each dNTP, 1.5 mM MgCl₂ (except marker D7Mit259, for which the optimal concentration was 2.5 mM), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 0.5 μ of Perfect Taq Predymerase (Central European Bioysstems, Prague, Czech Republic) and approximately 40 ng of tail DNA. PCR reaction was performed using the DNA Engine Dyad® Peltier Thermal Cycler (Bio-Rad, Hercules, CA), according to the following scheme: an initial hot start 3 min at 94°C, followed by 40 cycles of 94°C for 30 s for denaturing, 55°C for 60 s for annealing (except marker D7Mit259, for which optimal Ta = 52°C), 72°C for 60 s for elongation, and finally 3 min at 72°C for final extension. Each PCR product was electrophoresed in 3% agarose gel containing 80% of MetaPhor® Agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME) and 20% of UltraPure™ Agarose (Invitrogen, Carlsbad, CA) for 15 min to 2 h at 150 V.

Precision mapping of Tbbr2

To map precisely Tbbr2 on STS-derived segment of strain CsC-11 on proximal part of chromosome 12 [32] we used 8 microsatellite markers: D12Mit10a, D12Mit11, D12Mit209, D12Mit182, D12Mit104, D12Mit240, D12Mit170, Dtb (dystrobrevin, beta) and 4 SNPs; rs48212577, rs4229232, rs50154157 and rs50776991 (Generi Biotech, Hradec Králové, Czech Republic). The conditions of PCR reaction were as described in the section Genotyping of F2 mice.

Polymorphism of SNPs was tested by restriction analysis after PCR reaction using following restriction enzymes [New England BioLabs, Ipswich, MA]: HpyAV for rs48212577 (14.13 μl of PCR product, 2 U (1 μl) of HpyAv, 1.7 μl of 10X NEB buffer 4 [200 mM Tris-acetate, 500 mM Potassium Acetate, 100 mM Magnesium Acetate, 10 mM Dithiothreitol, pH 7.9], 0.17 μl of 10 mg/ml BSA (bovine serum albumin), 37°C, o/n); HinfI for rs4229232 (14.8 μl of PCR product, 5 U (0.5 μl) of HinfI, 1.7 μl of 10X NEB buffer 4, 37°C, o/n); BsmFI for rs50154157 (14.13 μl of PCR product, 2 U (1 μl) of BsmFI, 1.7 μl of 10X NEB buffer 4, 0.17 μl of 10 mg/ml BSA, 65°C, o/n), and Tsp509I for rs50776991 (14.8 μl of PCR product, 2 U (0.5 μl) of Tsp509I, 1.7 μl of 10X NEB buffer 4 [100 mM Bis-Tris-propane-HCl, 100 mM MgCl2, 10 mM Dithiothreitol, pH 7.0], 65°C, o/n). The products were electrophoresed in 3% agarose gel containing 80% of MetaPhor® Agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME) and 20% of UltraPure™ Agarose (Invitrogen, Carlsbad, CA) for 15 min to 2 h at 150 V.

Statistical analysis

For the strain pattern analyses, differences in survival after T. b. brucei infection were compared between the RC strains CsC-5, CsC-11, CsC-16 and CsC-20 and the parental strains BALB/c and STS by Kaplan-Meier estimator using the PROC LIFETEST procedure of the SAS 9.1 statistical package for Windows (SAS Institute, Inc., Cary, NC). The differences between strains BALB/c, STS and CsC-11 in splenomegaly, hepatomegaly and body weight change were evaluated by the analysis of variance (ANOVA) and Newman-Keuls multiple comparison test at 95% significance.

Differences between strains BALB/c, STS and CsC-11 in splenomegaly, hepatomegaly and body weight change were evaluated by the analysis of variance (ANOVA) and Newman-Keuls multiple comparison using the program Statistica for Windows 8.0 (StatSoft, Inc., USA). Strain and age were fixed factors and individual experiments were considered as a random parameter. The differences in parameters between strains were evaluated using the Newman-Keuls multiple comparison test at 95% significance. Differences between strains BALB/c, STS and CsC-11 in chemokine and cytokine levels were calculated by Mann Whitney U test.

Linkage of microsatellite markers with survival after T. b. brucei infection in F2 hybrids was examined by analysis of variance (ANOVA, PROC GLM statement of the SAS 8.2 for Windows (SAS Institute, Inc., Cary, NC)). Log_10 transformation was performed in order to obtain normal distribution. The effect
of each marker, sex and experiment on mouse survival was tested. Each individual marker and its interactions with other markers and sex or experiment were subjected to ANOVA. A backward elimination procedure [33] was used. The first round of the backward elimination procedure results in a list of significant markers and a list of interactions. This list (the markers and interactions with $P$ value smaller than 0.05) is the input for the second round of ANOVA and the marker (or interaction) bearing the highest $P$ value (if $P>0.05$) is eliminated. The backward elimination procedure was repeated till the final set of significant markers and interactions was obtained.

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

$$x_T = \frac{C + 2pGh(T)}{C}$$

where $G = 1.75$ Morgan (the length of the segregating part of the genome; 12.5% of 14 M); $C = 8$ (number of chromosomes segregating in cross between CcS-11 and BALB/c, respectively); $p = 1.3$ for $F_2$ hybrids; $h(T)$ = the observed statistic (F ratio).

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

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Results

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

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

We have compared splenomegaly, hepatomegaly, changes of body weight (Figure 2), and differences in cytokine and chemokine levels (Figure 3) after T. b. brucei infection. Female mice strains of BALB/c (11 infected tested 2nd day p.i., 22 infected tested 10th day p.i., 22 non-infected), STS (9 infected tested 2nd day p.i., 17 infected tested 10th day, 13 non-infected) and CcS-11 (14 infected tested 2nd day p.i., 25 infected tested 10th day p.i., 26 non-infected) were compared. Animals were intra-peritoneally inoculated with 2.5×10^4 bloodstream forms of T. b. brucei. Control, non-infected mice were kept in the same animal facility. Mice were killed 10 days after inoculation. The data show the means ± SD from three independent experiments. Asterisks indicate significant difference from BALB/c. doi:10.1371/journal.pntd.0001173.g002

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

Figure 3. Differences in chemokine levels in strains BALB/c, STS and CcS-11 after T. b. brucei infection. Female mice strains of BALB/c (11 infected tested 2nd day p.i., 22 infected tested 10th day p.i., 22 non-infected), STS (9 infected tested 2nd day p.i., 17 infected tested 10th day, 13 non-infected) and CcS-11 (14 infected tested 2nd day p.i., 25 infected tested 10th day p.i., 26 non-infected) were compared. Animals were intra-peritoneally inoculated with 2.5×10^4 bloodstream forms of T. b. brucei. Control, non-infected mice were kept in the same animal facility. Mice were killed 10 days after inoculation. The data show the means ± SD from three independent experiments. Asterisks indicate significant difference from BALB/c. doi:10.1371/journal.pntd.0001173.g003
levels (Figure 3) in females of background strain BALB/c, donor strain STS and RC strain CcS-11. Non-infected mice do not differ in spleen to body weight ratio (Figure 2A) and in changes of body weight (Figure 2C), whereas liver to body weight was higher in BALB/c than in both STS (P<0.0000001) and CcS-11 (P<0.0000001) (Figure 2B). Infection led to a significant enlargement of spleens (BALB/c: P = 0.000001; STS: P = 0.000004; CcS-11: P = 0.000001) and livers (BALB/c: P = 0.000001; STS: P = 0.00007; CcS-11: P = 0.000001) in all tested strains and to decrease of body weight (BALB/c: P = 0.000063; STS: P = 0.000044; CcS-11: P = 0.00037) in comparison with non-infected mice. BALB/c exhibited higher splenomegaly than STS (P<0.0000001) and CcS-11 (P<0.0000001) and also higher hepatomegaly than both STS (P<0.0000001) and CcS-11 (P<0.0000001). Differences in changes in body weight during the infection were observed between BALB/c and STS (P = 0.0000).

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

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

We examined survival after *T. b. brucei* infection in 169 F2 hybrids between the strains BALB/c and CcS-11. The strain CcS-11 differs from BALB/c in the genetic material at 8 chromosomes that were received from STS [32]. These differential STS-derived segments were genotyped in the F2 hybrid mice using 14 microsatellite markers. Statistical analysis of linkage revealed four genetic loci that influence survival after *T. b. brucei* infection. Two of these loci have individual effects (Table 1); the other two operate in mutual non-additive interaction (Table 2). The effects of all loci were more expressed in females than in males.

Two loci, *Tbbr1* (*Trypanosoma brucei* brucei response 1) linked to D3Mit45 (corrected *P* value = 0.0494 females; corr. *P* = 0.267 both sexes) and *Tbbr2* linked to D12Mit37 (corrected *P* value = 0.0224 females; corr. *P* value = 0.583 both sexes) have main effects on survival that are not dependent on or influenced by interaction with other genes (main effects) (Table 1, Figure 4 A,B,C,D). These loci have in CcS-11 an opposite effect on the studied trait. The homozygosity for the STS allele of *Tbbr1* (SS) determines about 4 days longer survival than homozygosity of the BALB/c allele (CC), whereas homozygosity for the STS allele of *Tbbr2* (SS) is associated with about three days shorter survival than the homozygosity of the BALB/c allele (CC). We have also observed a suggestive linkage of survival to D6Mit85 (corrected *P* value = 0.0542 females; corr. *P* = 0.0994 both sexes), heterozygotes had the shorter survival (Table 1).

*Tbbr3* linked to D7Mit282 influences survival in interaction with *Tbbr4* linked to D19Mit51 (corrected *P*<0.0332 females; corr. *P* = 0.0430 both sexes). F2 mice with homozygous BALB/c (CC) alleles at *Tbbr3* and STS (SS) alleles at *Tbbr4* or homozygous for STS allele at *Tbbr3* and homozygous for BALB/c alleles in *Tbbr4* have the shorter survival in comparison with other combinations of *Tbbr3* and *Tbbr4* STS and BALB/c alleles (Table 2, Figure 4 E,F). A suggestive linkage was detected in females in interaction of D6Mit85 and D19Mit60 (corrected *P* = 0.0555), shorter survival has been observed in mice heterozygous both in D6Mit85 and D19Mit60 (Table 2).

**Precision mapping of *Tbbr2***

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

**Discussion**

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

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

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

*F*2 hybrids between CcS-11 and BALB/c were tested. Means and standard errors of means of survival times and *P*-values were computed by analysis of variance. Logarithmic (Log 10) transformation was used to obtain normal distribution and the obtained values were retransformed after calculation. Number of tested mice is shown in brackets.

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The BALB/c genome that work in interaction with STS disease shorter survival after infection than either parent. The elements in STS and 87.5% genes of the background strain BALB/c and it has groups [15,16]. Donor strain STS does not differ in survival from T. b. brucei F2 hybrids between CcS-11 and BALB/c were tested. Means and standard errors

| A. Females |  |  |  |
|---|---|---|---|
| D19Mit51 (Tbbr4) | P = 0.0009 | Corrected P = 0.0032 |
| CC | CS | SS |
| D7Mit282 CC | 18.46 ± 1.32 | 18.79 ± 0.88 | 16.08 ± 0.74 |
| (Tbbr3) | (n = 6) | (n = 11) | (n = 9) |
| CS | 18.14 ± 0.85 | 17.77 ± 0.86 | 17.01 ± 0.81 |
| SS | 14.90 ± 1.06 | 17.33 ± 0.82 | 18.66 ± 0.88 |
| P = 0.0016 | Corrected P = 0.0555 |

| B. Both sexes |  |  |  |
|---|---|---|---|
| D19Mit60 | P = 0.0013 | Corrected P = 0.0430 |
| CC | CS | SS |
| D7Mit282 CC | 18.96 ± 1.36 | 16.68 ± 0.78 | 19.45 ± 1.39 |
| (Tbbr3) | (n = 5) | (n = 12) | (n = 7) |
| CS | 17.06 ± 0.76 | 15.05 ± 0.36 | 15.94 ± 0.77 |
| SS | 16.96 ± 1.20 | 20.13 ± 1.44 | 17.16 ± 0.78 |
| P = 0.0420 | Corrected P = NS |

| D19Mit60 | P = 0.0009 | Corrected P = 0.0032 |
| CC | CS | SS |
| D7Mit282 CC | 18.46 ± 1.32 | 18.79 ± 0.88 | 16.08 ± 0.74 |
| (Tbbr3) | (n = 6) | (n = 11) | (n = 9) |
| CS | 18.14 ± 0.85 | 17.77 ± 0.86 | 17.01 ± 0.81 |
| SS | 14.90 ± 1.06 | 17.33 ± 0.82 | 18.66 ± 0.88 |

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

The observations of progeny having a phenotype, which is beyond the range of the phenotype of its parents are not rare in traits controlled by multiple genes. Some F2 hybrids derived in cross between trypanotolerant African N’Dama (Bos taurus) and trypanosusceptible Kenya Boran (Bos indicus) cattle differed from both parents and contained less T. congolense parasites than any of them [37]. Similarly, mouse RC strain OcB-9 differs from both parental strains O20 and B10.O20 in response to alloantigens [38], several RC strains exhibit in some parameters higher susceptibility to Leishmania major than both parental strains BALB/c and STS [39], and analysis of gene expression from livers in chromosome substitution strains [background strain C57BL/6, donor strain A/J] revealed that only 438 out of 4209 expression QTLs were inside the parental range [40]. These observations are due to multiple gene-gene interactions of QTLs, which in new combinations of these genes in RC strains, F2 hybrids or in chromosomal substitution strains can lead to appearance of new phenotypes that exceed their range in parental strains. Also, with traits controlled by multiple loci, the parental strains often contain susceptible alleles at some of them and resistant on others, and some progeny may receive predominantly susceptible alleles from both parents.

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

**Susceptibility loci and potential candidate genes**

We have detected four loci that in the strain CsS-11 control survival after T. b. brucei infection and mapped them with a precision of 1 cM–25 cM (Tables 1, 2, Figure 5). Usually, a standard inbred-strain mapping experiment using F2 hybrids will map a QTL onto a 20- to 40-cM interval [41]. Using advanced intercross lines [20,22] the susceptibility loci Tw1 and Tw3c to T. congolense were mapped with a 95% confidence interval to 1.3 and 2.2 cM, respectively. In the RC strains the donor-derived segments of medium length (5–25 cM) comprise 54% of donor genome [42]. However, RC strains can carry on some chromosomes very short segments of donor strain origin. This feature of the RCS system allowed us previously to narrow the location of Lmr9 (Leishmania major response 9) on chromosome 4 to a short segment of 1.9 cM without any additional crosses [43]. The short length of this segment, which controls levels of serum IgE in L. major infected mice, enabled us to map a human homolog of this locus on human chromosome 8 and show that it controls susceptibility to atopy [44].

Our data show sex influence on survival as after correction for the genome-wide testing significance of the Tbbr loci was detected only in females or in the whole tested group. This observation can be related to the influential role of sex hormones in control of parasitic infections by their ability to modulate different compo-
nents of both the innate and adaptive immune responses [45,46]. Greenblatt and Rosenstreich [47] analyzed resistance of the 10 inbred mouse strains and two sets of F1 hybrids to infection with *T. b. rhodesiense*. C3H/HeN, C3H/HeJ, CBA/J, BALB/c and CBA/CaJ were highly susceptible, with mean survival times of less than 22 days, and did not exhibit differences in survival between males...

Figure 4. Differential survival of F2 hybrid mice after *T. b. brucei* infection. Mice were intra-peritoneally inoculated by 2.5 x 10^6 bloodstream forms of *T. b. brucei*. A. females and B. both sexes carrying BALB/c or STS homozygous alleles in *Tbbr1* (D3Mit45); C. females and D. both sexes carrying BALB/c or STS homozygous alleles in *Tbbr2* (D12Mit37); E. females and F. both sexes carrying interacting STS homozygous alleles in *Tbbr3* (D7Mit282) and BALB/c homozygous alleles in *Tbbr4* (D19Mit51) or BALB/c homozygous alleles in *Tbbr3* and heterozygotes in *Tbbr4*. n, number of mice. doi:10.1371/journal.pntd.0001173.g004

b. rhodesiense, C3H/HeN, C3H/HeJ, CBA/J, BALB/c and CBA/CaJ were highly susceptible, with mean survival times of less than 22 days, and did not exhibit differences in survival between males.
and females, whereas in more resistant strains CBA/N, A.CA, C57BL/6J, C57BL/KsJ, C57BL/10SnJ, (BALB/c x C57BL/6)F1 and (C57BL/6 x BALB/c)F1 female mice were more resistant than males. These data support the finding of different genetic regulation of susceptibility to *Trypanosoma brucei* in males and females in certain genetic combinations. Genes controlling infections that
appear to be sex dependent have been observed also with other pathogens. For example, Rnk4 (resistance to mouse pox 4) controls susceptibility to ectromelia virus in female mice only [48] and H11 (herpes resistance locus) exhibits higher influence on susceptibility to Herpes simplex virus in male than in female mice [49]. Sex specific QTLs influence also susceptibility to Thielers murine encephalomyelitis virus-induced demyelination; loci Tmved7 and B affect male mice only, whereas locus Tmved9 controls susceptibility only in females. Locus Tmved6 operates both in females and males, but it has an opposite effect on disease susceptibility in males and females [50]. Lmr20 influenced IgE level in L. major infected females, but not in males [35]. QTLs Cnes1 and Cnes2 were associated with high pulmonary Cryptococcus neoformans burden in females, whereas Cnes3 was associated with fungal pulmonary burden in male mice [51]. QTL on chromosome 17 controls susceptibility only in female mice [52]. In humans, for has much stronger effect in males, whereas QTL on chromosome 20 controls susceptibility to Theiler's murine encephalomyelitis virus-induced demyelination: loci Tbbr1 and Tbbr4 operate both in females and males, but it has an opposite effect on disease susceptibility in males and females [53]. Polymorphism (rs2069885) has an influence on IgE level in infected individuals with Chlamydia pneumoniae, but it has an opposite effect on disease susceptibility in males and females [54]. Pomp3 (pro-opiomelanocortin-alpha) [56,57], Atyd3 (adenylate cyclase 3) [58], and N001 (nuclear receptor coactivator 1) [59] in immune response against Trypanosoma.

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

| Gene                        | ID                  | Liver | Spleen | Brain |
|-----------------------------|---------------------|-------|--------|-------|
| Gm11061, predicted gene 11061 | MGI:3779285         | NT    | NT     | NT    |
| Dmnt3a, DNA methyltransferase 3A | MGI:1261827         | YES   | <M     | >M    |
| Gm10485, predicted gene 10485 | MGI:3641689         | NT    | NT     | NT    |
| Pomic, pro-opiomelanocortin-alpha | MGI:97742         | NO    | <M     | YES   |
| Efr3b, EFR homolog B (S. Cerevisiae) | MGI:2444851     | NO    | >M     | YES   |
| Dnsg27, Dnaq (Hsp40) homolog 8, subfamily C, member 27 | MGI:2443036     | NO    | <M     | YES   |
| Adcy3, adenylate cyclase 3 | MGI:99675           | YES   | <M     | >M    |
| Cenpo, centromere protein O | MGI:1923800         | YES   | <M     | >M    |
| Jc10017P0909ik, RIKEN cDNA Jc140017P09 gene | MGI:1916999 | YES   | >M     | YES   |
| Ncoa1, nuclear receptor coactivator 1 | MGI:1276523     | YES   | <M     | YES   |
| Gm3613, predicted gene 3613 | MGI:3781789         | NO    | NT     | NT    |
| Gm3620, predicted gene 3620 | MGI:3781796         | NO    | >M     | NO    |
| Gm3625, predicted gene 3625 | MGI:3781801         | NO    | NT     | YES   |
| Rtn2, intersectin 2 | MGI:1338049         | YES   | <M     | >3xM  |
| 4930417G10rik, RIKEN cDNA 4930417G10 gene | MGI:1922105     | NO    | >M     | NO    |
| A83093124ik, RIKEN cDNA A83093124 gene | MGI:2442121     | YES   | >M**   | YES   |
| Pfn4r, profilin family member 4 | MGI:1920121         | NO    | <M**   | NT    |
| Gm6682, predicted gene 6682 | MGI:3647156         | NT    | >M     | NT    |
| 6100009D07ri, RIKEN cDNA 6100009D07 gene | MGI:1913305     | YES   | <M     | >M    |
| Fkbp1b, FK506 binding protein 1b | MGI:1336205        | NO    | <M     | NO    |
| Bc060828i, cDNA sequence Bc060828i | MGI:3040699     | YES   | >3xM   | YES   |
| Mfd1a2b, major facilitator superfamily domain containing 2B | MGI:3583946     | NO    | <M     | NO    |
| Ubxn2a, UBX domain protein 2A | MGI:2442310         | YES   | >M     | YES   |
| Atad2b, ATPase family, AAA domain containing 2B | MGI:2444798     | YES   | <M     | YES   |
| Klh29, kelch-like 29 (Drosophila) | MGI:2683857        | NO    | <M     | >3xM  |
| 2810032G03rik, RIKEN cDNA 2810032G03 gene | MGI:1919919     | NO    | >M     | YES   |

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

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lineage antigen, present on both B-1 and B-2 cells [61]. It was shown that in murine experimental \( T. brucei \) trypanosomiasis, B-cells were crucial for periodic peak parasitemia clearance and survival of host [16]. CD5+ subpopulation of B-1 cell has been found to be stimulated by different \( Tryptosoma \) species: \( T. cruzi \) [62], \( T. b. rhodesi \) and \( T. congolense \) [64]. These B-cells were the main source of antibodies reactive with non-parasite antigens in \( T. congolense \)-infected cattle [64]. However, genes that are presently not considered as possible candidates might cause the effects of some or all \( Tbrb \) loci. Moreover, not only genes, but also noncoding RNAs in \( Tbrb \) loci region may influence the outcome of infection [65].

Are \( Tbrb \) loci involved in control of other pathogens?

Some genes, for example \( Slc11a1 \) (solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1) or \( Lyst \) (lysosomal trafficking regulator) \( / \) beigy have been found to control susceptibility to several pathogens (reviewed in ref [29]). \( Tbrb2 \) might be potentially involved also in control of \( Leishmania \) major, as it overlaps with locus \( Lmu22 \) (\( Leishmania \) major response 22), which in interaction with \( Lmu5 \) controls serum \( IL-1 \) in \( L. major \) infected mice [35], whereas \( Tbrb3 \) on chromosome 7 maps near to \( Ity7 \) (immunity to \( S. tryphaimum \) 7) [66].

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

In summary, this study represents the first definition of genetic loci controlling susceptibility to \( T. brucei \) infection. One of them, \( Tbrb2 \) is precisely mapped to the segment that contains only 26 genes, which will facilitate the identification of the candidate gene. \( T. brucei \) subspecies cause sleeping sickness in humans and affect also all livestock, with particularly severe effects in horses and dogs [1]. Thus, the definition of genes controlling anti-parasite responses might also permit a better understanding of pathways and genetic diversity underlying the disease phenotypes in humans and domestic animals.

Supporting Information

Figure S1 Differences in levels of CCL4/MIP-1\( \beta \), CCL5/ RANTES, and TNF-\( \alpha \) between infected and non-infected mice. Female mice strains of \( BALB/c \) (11 infected tested 2\( \text{nd} \) day p.i., 22 infected tested 10\( \text{th} \) day p.i., 22 non-infected), \( STS \) (9 infected tested 2\( \text{nd} \) day p.i., 17 infected tested 10\( \text{th} \) day, 13 non-infected) and \( CeS-11 \) (14 infected tested 2\( \text{nd} \) day p.i., 25 infected tested 10\( \text{th} \) day p.i., 26 non-infected) were compared. Animals were intra-peritoneally inoculated with \( 2.5 \times 10^7 \) bloodstream forms of \( T. brucei \). Control, non-infected mice were kept in the same animal facility. Mice were killed 10 days after inoculation. The data show the means \( \pm \) SD from three independent experiments. (TIF)

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

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

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Author Contributions

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

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