Supporting Online Material for

Leishmania RNA Virus Controls the Severity of Mucocutaneous Leishmaniasis

Annette Ives, Catherine Ronet, Florence Prevel, Giulia Ruzzante, Silvia Fuertes-Marraco, Frederic Schutz, Haroun Zangger, Melanie Revaz-Breton, Lon-Fye Lye, Suzanne M. Hickerson, Stephen M. Beverley, Hans Acha-Orbea, Pascal Launois, Nicolas Fasel,* Slavica Masina

*To whom correspondence should be addressed. E-mail: nicolas.fasel@unil.ch

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Materials and Methods

Mice strains

5 to 6 week old C57BL/6 and BALB/c mice were purchased from Harlan Laboratories (Netherlands). MyD88$^{-/-}$, TLR7$^{-/-}$, and TLR9$^{-/-}$ mice were obtained Prof. S. Akira (Osaka University, Japan) via P. Launois (WHO-IRTC, Lausanne, Switzerland), or P. Romero (Ludwig Institute for Cancer, Lausanne, Switzerland) for TLR3$^{-/-}$ mice. TRIF$^{\Delta LPS2}$ were obtained via B. Ryffel, (CNRS, Orléans, France)(*32*). The mice were bred and maintained at the animal facility of the Center of Immunity and Immunology, Lausanne (Switzerland) under pathogen free conditions. The mice and all experiments performed adhered to the guidelines set by the State Ethical Committee for the use of laboratory animals. All mutant and deficient mice were crossed onto a C57BL/6 background for at least eight generations.

Parasite and cell culture

L. guyanensis clones either non-metastatic (*L.g.*M-: Lg03, Lg17) or metastatic (*L.g.*M+: Lg13, Lg21) were derived from metastatic *L. guyanensis* M5313 parasites (*L.g.*M5313(M+),WHI/BR/78/M5313) from CIDEIM (Centro Internacional de Entrenamiento e Investigaciones Médicas (*14*). Human isolates of *L. guyanensis* Lg1398 (MHOM/BR/1989/IM3597) and Lg1881 (MHOM/BR/1992/IM3862) were obtained from the CLIOC (Coleção de *Leishmania* do Instituto Oswaldo Cruz, Brazil), and *L. major* LV39 (MRHO/SU/59/P) and IR75 (MRHO/IR/75/ER) were obtained from WHO (World Health Organization). Parasites were cultured at 23^{o}C in M199 medium (Gibco®) consisting of 10% FBS, 1% penicillin/streptomycin, and 5% Hepes (Sigma-Aldrich®), or on NNN media or grown in freshly prepared Schneider's Insect Medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1% penicillin/ streptomycin (Gibco®).

The LRV-bearing strain of *L. guyanensis* M4147 (MHOM/BR/75/M4147-*L.g.*M4147-LRV^{high}) and a virus free derivative (M4147/pX63-HYG-*L.g.*M4147-LRV^{neg}) expressing luciferase were described previously (*29, 30*). These lines contain the *LUC* gene integrated stably into the small subunit gene of the ribosomal RNA locus, yielding the LRV+ line M4147/*SSU:IR2SAT-LUC*(b) and the LRV- line M4147*/pX63HYG*/*SSU:IR2SAT-LUC*(b). These parasites express high levels of luciferase (5 x 10^7 photons/sec/1 x 10^6 parasites, measured when cells were in logarithmic growth phase).

In general, the parasites were maintained in culture for a maximum of 7 passages following either isolation from hamsters for all *L. guyanensis* M5313 derived parasites, from mouse footpads for *L. major* strains and *L.g.*M4147 strains, or after receipt from the collection banks. All mammalian cells were cultured in complete DMEM (Gibco®) with 10% FBS, 1% penicillin/streptomycin, and 1% Hepes (Sigma-Aldrich®).

Macrophage infection experiments

Bone marrow cells were extracted from the femurs and tibias of naïve mice. The extracted cells were differentiated into bone marrow derived macrophages (BMMφ) for 5 days using complete DMEM supplemented with L929 conditioned media at 37^0C. Differentiated BMMφ were coated onto microtiter plates and infected (1:10) with stationary phase *Leishmania* parasites for 2, 6 or 24hrs. BMMφ were also stimulated with LPS (Sigma-Aldrich®), Poly I:C (Invivogen), or CpG (Invivogen) at 200 or 100ng/ml, 8 or 1 µg/ml and 5µM respectively or pretreated with Chloroquine (20µM), or Cytochalsin D (40µM), for 2 hours and 1 hour respectively (Sigma-Aldrich®) (*18, 33*). Supernatants were collected and cells were lysed in RLT® (Qiagen) for RNA extraction. Infectivity of parasites was controlled by infection on culture slides stained with Diff-Quick® (Dade Behring) and the infectivity, and parasite burden of the different *Leishmania* parasites into BMMϕ was calculated. Briefly, 750 BMMϕ were counted in 3 randomly selected microscope fields of view and the average percentage infectivity and number of infected BMMϕ was calculated.

DNA Microarray

Three biologically independent experiments were performed. For each experiment transcript levels were compared from RNA preparations of uninfected BMMf's or BMMf's infected with either *L.g.* M+ (Lg13) or *L.g.* M-(Lg17) parasites. In addition, a dye-swap hybridization was performed for each comparison. RNA was purified by RNAeasy Mini Kit (Qiagen™), and the quality and quantity were verified by the Agilent Technologies (Germany) 2100 bioanalyzer and RNA 6000 Nano Assay LabChip® kit. Mouse cDNA was produced and printed on glass-slide microarrays by the DNA Array Facility Lausanne (DNA Array Facility Lausanne (DAFL), Switzerland). The 17k mouse cDNA microarray was made using the 15'000 gene clone set (NIA 15k cDNA set) available from the National Institute on Aging (NIA, USA). These cDNA clones are derived from embryonic and fetal mouse tissues. Additional 1400 cDNA clones were added from genes not contained in the NIA collection, containing both known genes and ESTs (GEO database: GSE21418). Briefly, cDNA was synthesized from 5 µg of RNA by direct incorporation of Cy3 or Cy5 fluorophore-labeled dCTP using random primers (Invitrogen) mediated by the Superscript II reverse transcriptase. For each labeling reaction, reference control RNA (2µl Alien spikes pool and 2µl Arabidopsis spikes pool obtained from the DAFL) was added for data normalization. The labeled probes were purified using the MiniElute™ PCR Purification kit (Qiagen), and mixed then concentrated using Millipore Microcon YM-30 columns. For hybridization, Cy3 and Cy5 labeled cDNA were mixed together, and loaded onto the glass-slides. Glass-slides were then scanned using an Agilent Technologies microarray scanner. The resulting TIF images were analyzed using GenePix Pro software (Axon Instruments, USA). Data analysis was performed using R statistics software (http://www.r-project.org/), Cy5 (red) and Cy3 (green) signal intensities were used to calculate M and A values for the array spots. Genes that were at least 1.5 fold over or under-expressed and with a p-value <0.05 were considered as differentially expressed. Statistical significance

was calculated after standardization between the slides using the Limma statistical software package. Data analysis, quality assessment and normalization were performed by the DAFL. These resulting differentially expressed genes were then further analyzed using Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

***Isolation of RNA and cDNA Synthesis from macrophages* for Real time PCR**

For all experiments, RNA was isolated with RNAeasy Mini Kit (Qiagen), and quantified by using a NanoDrop® ND-100 Spectrophotometer (NanoDrop technologies Inc.). cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen™), followed by purification using the QIAquick™ PCR purification Kit (Qiagen). Gene expression levels were analyzed using quantitative Real Time PCR (qRT-PCR) with the LightCycler480® system (Roche Applied Science). Unless stated otherwise, gene specific primers were designed for this study using the LightCycler® Probe Design Software 2.0 and synthesized by Microsynth, Switzerland. Cxcl10; 5'-CTT GAA ATC ATC CCT GCC AC, and 5'-CGC TTT CAT TAA ATT CTT GAT GGT C, Ccl5; 5'-TCT CCC TAG AGC TGC CT, and 5'-TCC TTG AAC CAA CTT CTT CTC TG, Il6; 5'-TCC AGT TGC CCT CCT GGG AC, and 5'-GTG TAA AGC CTC CGA CTT C, Tnfa; 5'-CAT CTT CTC AAA ATT CGA GTG ACA A and 5'- TGG GAG TAG ACA AGG TAC AAC CC (*34*), Ifnb: 5'-AAC CTC ACC TAC AGG GC, and 5'-CAT TCT GGA GCA TCT CTT GG, and Tbp: 5'-CCG TGA ATC TTG GCT TA AAC and 5'-TCC AGT ACT GAA AAT CAA CGA. For amplification, the LightCycler® FastStart DNA Master SYBR Green I kit (Roche Applied Science) was used. The relative gene expression levels were quantified in duplicate for each sample in comparison to the Tbp reference gene. Analysis and acquisition of real time data was executed by the LightCycler software 1.5 (Roche Applied Science) and Qbase software (Biogazelle) using the $2^{-\Delta\Delta CT}$ method.

Analysis of cytokines and chemokines by ELISA

Supernatants from the infection experiments were analyzed in duplicate by ELISA. CXCL10, CCL5, and TNFα kits were purchased from R&D systems, IL6 (ebioscience) and IFNβ (PBL, Interferon Source) and were read on a Synergy™ HT Multi-Mode Plate Reader (Biotek Instruments, Switzerland). Results were expressed as the concentration of secreted protein above the unstimulated BMMφ control.

Nucleic acid extraction and LRV1 detection in Leishmania promastigotes

Parasites in PBS were lysed with 10% Sarcosyl (Sigma-Aldrich®), and treated with bovine pancreas derived RNAse (ssRNAse-Roche) and Proteinase K (Roche) for 2 hours at 37°C. Nucleic acids were extracted using Biophenol/chloroform/Isoamyl alcohol (Biosolve), precipitated with 3M sodium acetate in 75% ethanol, and resuspended in TE. Total RNA was extracted using TRIzol® reagent (Invitrogen ™). Where required, nucleic acids were treated with RQ1 DNAse (Promega), and/ or RNAse III (New England BioLabs) according to

manufacturer's instructions. Nucleic extracts were quantified using ND-100™ and electrophoresed on 1% agarose gels with *Lambda* DNA/EcoR1 + HindIII (Promega) as a marker. For purification of LRV1 dsRNA the 5.3 kb band was gel excised, purified by phenol/chloroform, and resuspended in LAL (endotoxin-free) reagent water (Promega). Reverse transcription of RNA into cDNA was performed as previously mentioned. qRT-PCR amplifications used LRV1 specific primers: 5'-CTGACTGGACGGGGGGTAAT-3' and 5'-CAAAACACTCCCTTACGC-3' and Kmp11 specific primers: 5'-GCCTGGATGAGGAGTTCAACA-3' and 5'-GTGCTCCTTCATCTCGGG-3' as described previously. Amplified DNA was excised from the gel, purified and sent to Fasteris SA for sequencing. The sequence homology of the LRV PCR was compared to reference sequences using Bioedit Software (Ibis Biosciences).

PCR amplifications were performed as follows: 50^0 C for 2 min and 95^0C for 10 sec then followed by 40 cycles of 95^0 C for 15 sec, 60^0 C for 1min. The LRV1-4 primers used were SMB2472/2473 set A (5'-GCATACCGTTTTGAGTGGAC and 5'-GTTTCAATCATTGGCTGACA respectively) or SMB3850/3851 set B (5'-TGTTACTTACCCTACGACTC and 5'-TGTGTAAGAAGTCAACT, respectively). Controls containing the same amount of RNA but lacking reverse transcriptase or template were used to rule out DNA or other contamination.

Mouse infection and parasite quantification

3 x 10^6 parasites of *L.g.*M-(Lg17), *L.g.*M5313(M+), *L.g.*M4147-LRV^{high}, *L.g.*M4147-LRV^{neg} were infected into the base of the hind footpads. Footpad swelling was measured weekly post infection using a Vernier caliper. For experiments with the *L.g.*M5313 strains parasites were quantified using the standard curve real time PCR quantification method using *Leishmania* Kmp11 specific primers on cDNA reverse transcribed from total RNA extracted from footpad lysates. Infection *in vivo* with luciferase expressing parasites *L.g.*M4147 (LRV^{high}, and LRV^{neg}) was analyzed with the In Vivo Imaging System (IVIS Lumina II, Xenogen) at the Cellular Imaging Facility (CIF, University of Lausanne). Mice were injected intra-peritoneally with 150 mg/kg D-luciferin (Xenogen) 10 min before imaging, anesthetized with isofluorane during imaging and the photons emitted from mice was quantified using the LivingImage version 3.2 software (Caliper Life Science). Parasite burden was expressed as photons per second emitted from *L.g.*M4147 infected mice footpad lesions normalized against the background fluorescence of uninfected mice.

Statistical test

All experiments had statistical significance determined at $p \leq 0.05$, or $p \leq 0.01$ using the Student's *t* test.

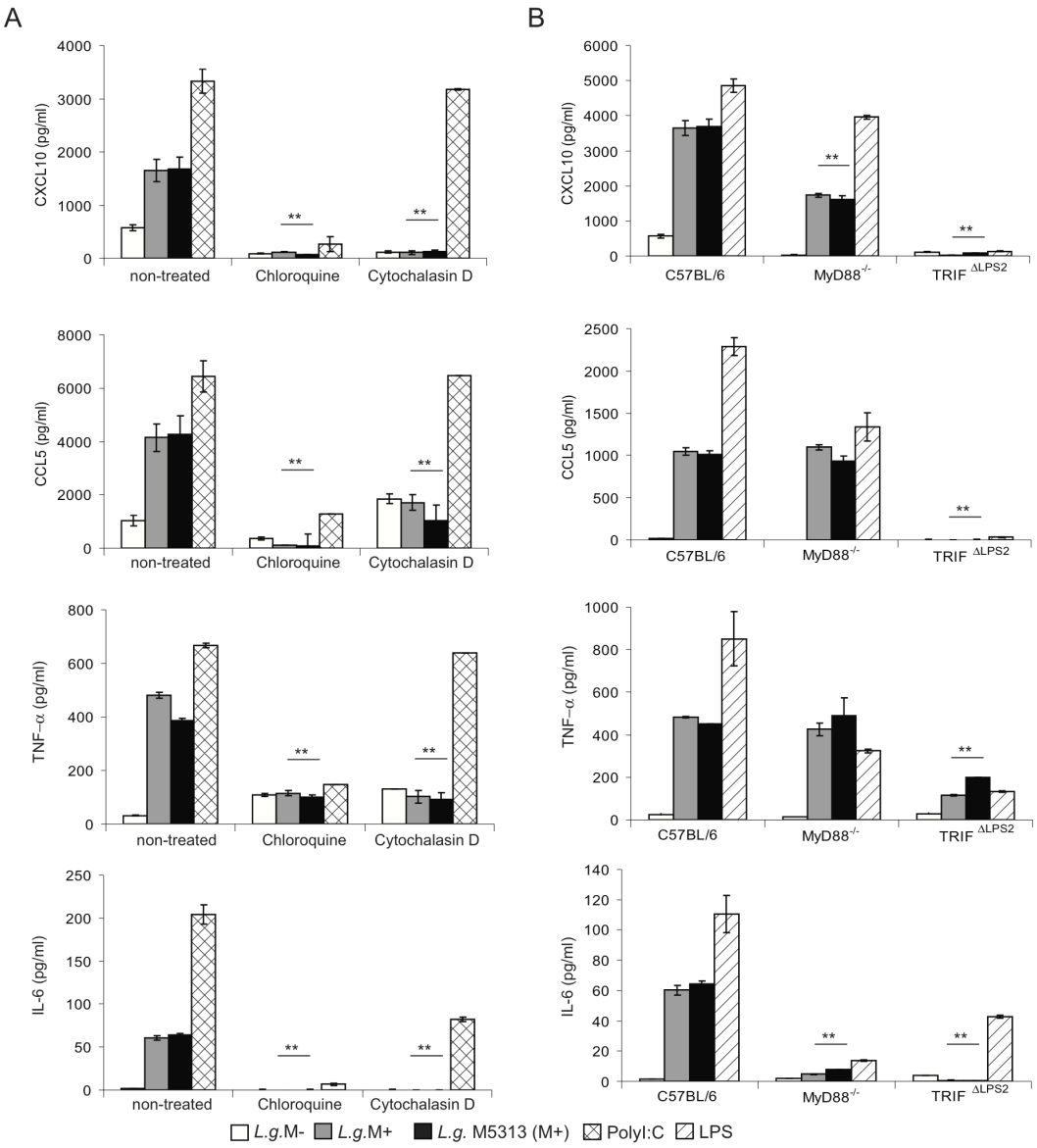

Fig. S1: Secretion of cytokines and chemokines by macrophages following *L.g.*M+ infection requires internalization, and endosomal recognition of an RNA motif by the TRIF-dependant TLR signaling pathway. Secreted cytokine and chemokine proteins were determined in macrophages infected 1:10 with *L.g.* parasites for 6 hours. Protein secretion levels were compared in wild-type, untreated C57BL/6 macrophages versus those pretreated with either chloroquine (20µM, 2 hours), or cytochalasin D (40µM, 1 hour) (A) or compared, to MyD88$^{-/-}$, and TRIF^{DLPS2} macrophages (B). LPS and/or Poly I:C at 200ng/ml, and 8µg/ml respectively were included. Data reflects at least 3 independent experiments, with mean ± SD protein concentration expressed above an unstimulated control, and statistical significance determined at (*) $\rho \leq 0.05$, and (**) $\rho \leq 0.01$.

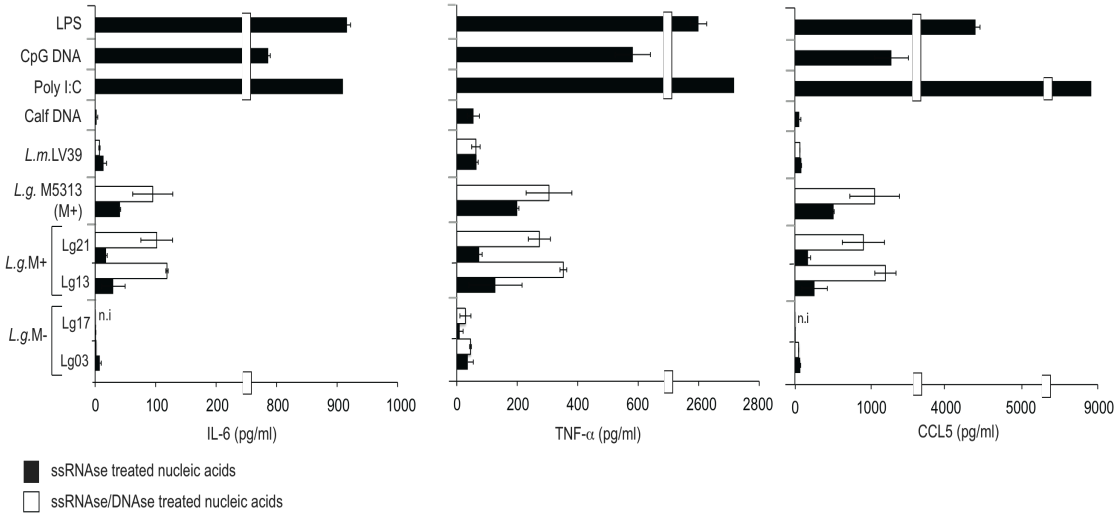

Fig. S2: Macrophages recognize a nucleic-acid-derived motif present in *L.g.* M+ parasites. BALB/c macrophages were treated with ssRNAse treated nucleic acids (5µg/ml) isolated by phenol/chloroform from *Leishmania* parasites or with ssRNAse and DNAse digested for 6 hours. Included within these experiments were controls of calf thymus DNA (5µg/ml), Poly I:C (2µg/ml), CpG (2µM), and LPS (100ng/ml). Data reflects at least 2 independent experiments, with mean ± SD protein concentration expressed above unstimulated control. n.i denotes not induced.

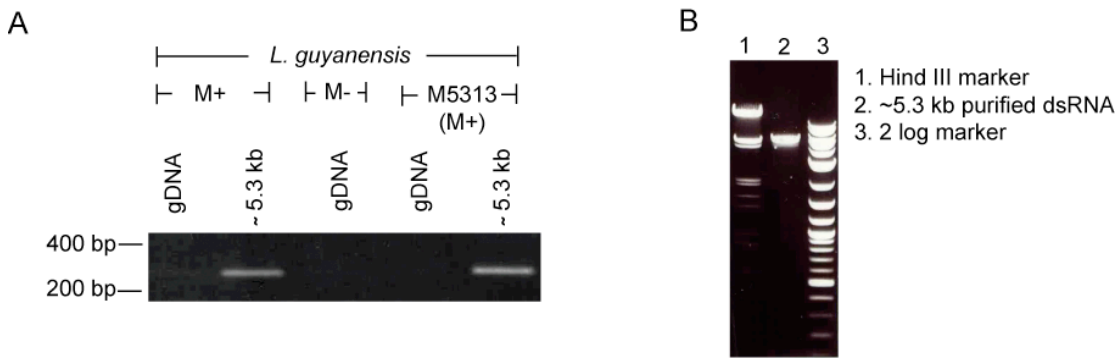

Fig. S3: Quality control and purification of LRV1 dsRNA from *L.g.*M5313 (M+). **(A)** Genomic DNA (gDNA) and ~ 5.3kb LRV1 dsRNA bands were visualized, and extracted from a 1% agarose gel, following ssRNAse treated total nucleic acids from stationary phase promastigotes of *L.g.* M+ (Lg13), *L.g.*M- (Lg17) and *L.g.*M5313(M+). The nucleic acids were extracted by phenol-chloroform, reverse transcribed, amplified by PCR using LRV1 specific primers and LRV1 specific products were visualized on a 1% agarose gel with the Log2 molecular marker. **(B)** Purity of the gel extracted ~5.3 kb band corresponding to LRV1 dsRNA was confirmed on a 1% agarose gel; HindIII: Lambda DNA/EcoRI + HindIII molecular weight marker, and 2 log following purification by phenol-chloroform.

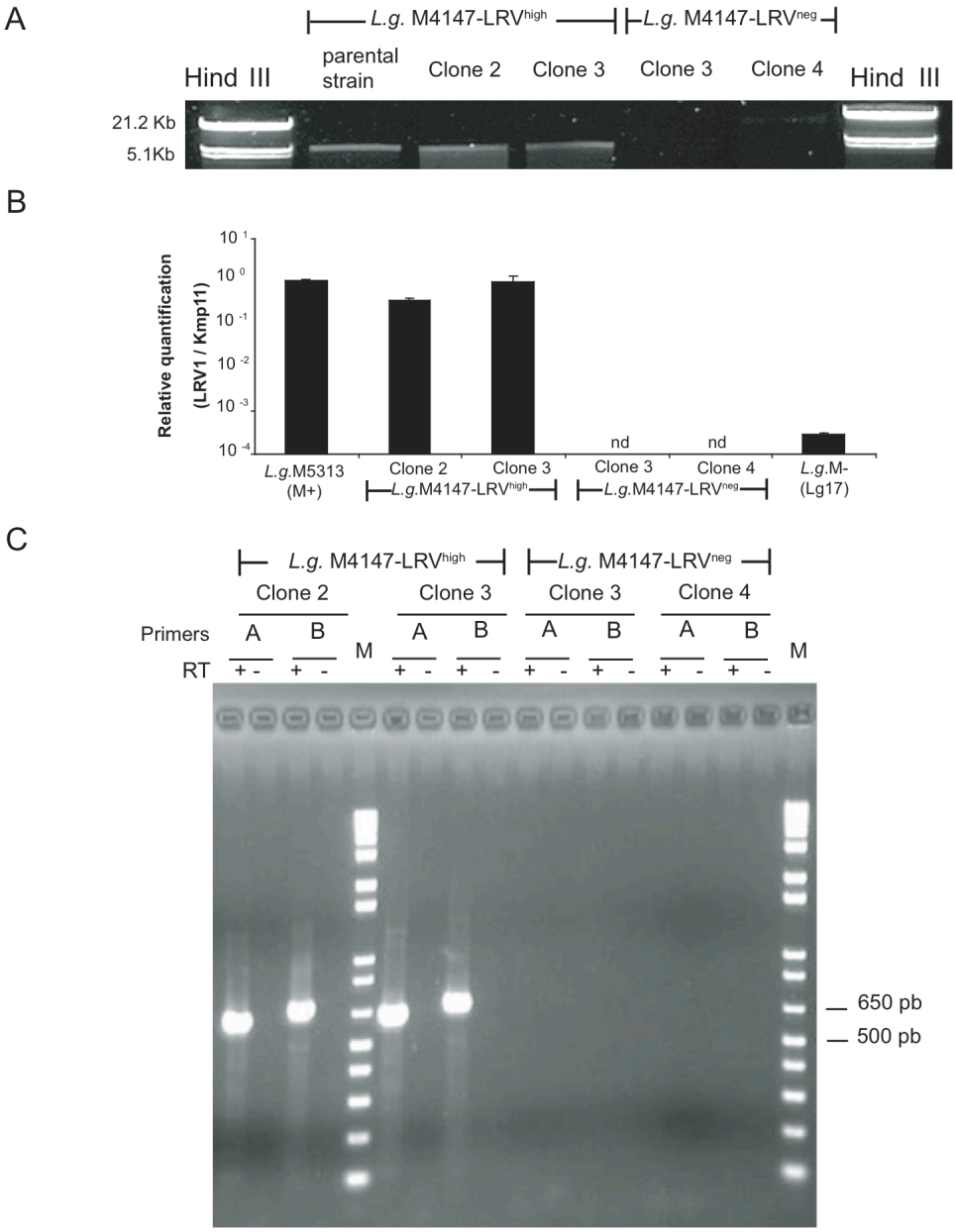

Fig. S4: Determination of the presence or the absence of LRV1-4 virus in *L.g.*M4147 and in two independent clones of *L.g.*M4147-LRV^{high} and isogenic virus-free derivative *L.g.*M4147-LRV^{neg}. **(A)** ssRNAse treated nucleic acids were treated with DNAse and the presence of the 5.3 kb LRV1 dsRNA band visualized by gel electrophoresis. Nucleic acids were separated on 1% agarose gels; HindIII: Lambda DNA/EcoRI + HindIII marker. **(B)** LRV1 virus relative quantification by qRT-PCR using Kmp11 as a reference gene; significance determined comparing relative LRV1 levels between *L.g.*M5313(M+) *L.g.*M-(Lg17), *L.g.*M4147LRV^{high} and *L.g.*M4147LRV^{neg}. **(C)** Analysis of two lines of *L. guyanensis* M4147 (*L.g.*M4147LRV^{high}) and its isogenic virus-free derivative (*L.g.*M4147LRV^{neg}). RT-PCR reactions were performed with LRV1-4 set A or set B; M, molecular size marker. n.d denotes not detected.

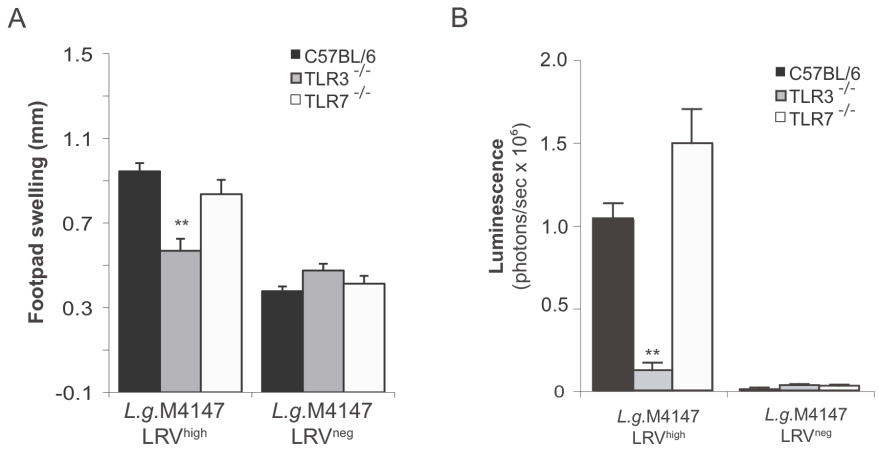

Fig. S5: TLR3$^{-/-}$ mice infected with *L.g.*M4147-LRV^{high} parasites have less disease pathology when compared with WT C57BL/6. Footpads of mice (n≥ 5) were infected with 3x10^6 parasites. **(A)** Footpad swelling, and **(B)** parasite burden were determined at 4 weeks post infection. Parasite burden was determined using relative luminescence. Results are expressed as mean ± SEM of all mice infected per group, with statistical significance at * p≤ 0.05, and ** p≤ 0.01.

Table S1. Infection rates of macrophages with *Leishmania* parasites and number of parasites per infected macrophages at 6 hrs post-infection. Macrophages immobilized onto a 6 well microscope culture slide were infected 1:10 with stationary phase *Leishmania* promastigotes for 6 hrs, and stained with Diff-Quick. The percent infection and number of parasites per infected cell of 750 counted macrophages was calculated. Results are expressed as mean ± standard deviation of 3 different microscope fields of view.

		Infection rate (%)	No. parasites per infected macrophages
Leishmania parasites infected into C57BL/6 macrophages			
*L.g.*M-	Clone Lg03	88.04 ± 5.03	5.90 ± 2.39
	Clone Lg17	92.15 ± 2.10	7.10 ± 0.88
*L.g.*M+	Clone Lg13	93.83 ± 4.60	7.63 ± 3.06
	Clone Lg21	88.77 ± 8.28	6.72 ± 1.85
	M5313	94.93 ± 4.30	10.24 ± 6.22
L.major	LV39	93.67 ± 3.94	6.51 ± 1.89
L.g.	M4147LRV^{high}	94.87 ± 2.10	5.75 ± 1.43
	M4147LRV^{neg}	93.3 ± 3.05	4.71 ± 1.17
Macrophages infected with *L.g.*M5313 (M+)			
C57BL/6 (Wildtype)		90.46 ± 7.11	7.6 ± 3.0
TLR3$^{-/-}$		88.66 ± 1.44	5.6 ± 2.5
TLR7$^{-/-}$		88.64 ± 5.71	5.9 ± 2.6
TLR9$^{-/-}$		89.49 ± 6.97	8.1 ± 3.0
TRIF$^{\Delta LPS2}$		91.44 ± 2.95	6.3 ± 1.7
Myd88$^{-/-}$		91.99 ± 4.54	6.8 ± 2.7

Supplementary material references

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