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Leishmania RNA Virus Controls the Severity of Mucocutaneous Leishmaniasis

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Abstract

Mucocutaneous leishmaniasis is caused by infections with intracellular parasites of the *Leishmania Viannia* subgenus, including *Leishmania guyanensis*. The pathology develops after parasite dissemination to nasopharyngeal tissues, where destructive metastatic lesions form with chronic inflammation. Currently, the mechanisms involved in lesion development are poorly understood. Here we show that metastasizing parasites have a high *Leishmania RNA virus*-1 (LRV1) burden that is recognized by the host Toll-like receptor 3 (TLR3) to induce proinflammatory cytokines and chemokines. Paradoxically, these TLR3-mediated immune responses rendered mice more susceptible to infection, and the animals developed an increased footpad swelling and parasitemia. Thus, LRV1 in the metastasizing parasites subverted the host immune response to *Leishmania* and promoted parasite persistence.

Leishmania parasites are obligate intracellular protozoan parasites transmitted to the mammalian host by the bite of an infected sand fly, where they predominantly infect macrophages. In Latin America, leishmaniasis caused by the *Leishmania Viannia* (*L. Viannia*) subgenus is endemic, causing cutaneous (CL) and mucocutaneous (MCL) leishmaniasis (1). Clinical MCL involves parasitic dissemination to the nasopharyngeal areas of the face, leading to destructive metastatic secondary lesions and hyperinflammatory immune responses (2–4). About 5 to 10% of individuals asymptomatic or with resolved CL lesions may develop MCL (1, 5, 6).

MCL development is associated with persistent immune responses showing proinflammatory mediator expression with high tumor necrosis factor α (TNF- α), CXCL10, and CCL4; a mixed intralesional T helper 1 (T_H1)/T_H2 phenotype; and elevated cytotoxic T

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Supporting Online Material

www.sciencemag.org/cgi/content/full/331/6018/775/DC1

Materials and Methods

Figs. S1 to S4

Table S1

References

cell activity (7–10). In addition to parasite-derived virulence factors, host genetics [such as polymorphisms for TNF- α and interleukin-6 (IL-6)] and immune status appear to influence MCL development (11, 12).

Hamsters infected with *L. Viannia* parasites isolated from human MCL lesions reproduce the metastatic phenotype with primary and secondary lesion development (13). Using this model, we characterized clones derived from the metastasizing *L. guyanensis* WHI/BR/78/M5313-*L.g.*M5313(M+) strain as metastatic (*L.g.*M+) or nonmetastatic (*L.g.*M-) after infection, depending on their ability to reproducibly develop secondary metastatic lesions (14). Previously, we showed that *L.g.*M+ clones derived from *L.g.*M5313 were more resistant to oxidative stress than *L.g.*M- clones and persisted in activated murine bone-marrow-derived macrophages despite their elevated nitric oxide levels (15).

On the basis of these observations, we hypothesized that *L.g.*M+ and *L.g.*M- parasites differentially modulate the host macrophage responses. Using DNA microarrays, we identified differential gene expression between uninfected macrophages and *L.g.*M+(1672) or *L.g.*M-(1513) infected macrophages, and *L.g.*M- directly compared to *L.g.*M+ (294) infected macrophages. Statistical significance was determined at ≥ 1.5 -fold, $P \leq 0.05$. We focused on genes involved in the immune response because of their relevance in MCL pathology.

In vitro, infected macrophages expressed significantly greater amounts of chemokines and cytokines CCL5, CXCL10, TNF- α , and IL-6 after infection with *L.g.*M+ parasites compared with *L.g.*M- parasites or *L. major* LV39 (Fig. 1, A and B) (16). We observed similar increased cytokine and chemokine expression after infection with *L.g.* from human MCL lesions (h-MCL-Lg1398) as compared to cytokine and chemokine expression during *L.g.* infection from human CL lesions (h-CL-Lg1881) (Fig. 1C). Thus, the elevated cytokine and chemokine levels after macrophage infection are associated with metastasizing parasites.

Leishmania parasites enter the macrophage endosomal compartment and form a phagolysosome (17). Pretreatment of macrophages with chloroquine, which induces vacuolar alkalinization and impairs recognition of pathogen-derived motifs by cells (18), or cytochalasin D, which inhibits parasite phagocytosis by inhibiting actin polymerization (19), showed that *L.g.*M+ parasite-dependent induction of proinflammatory mediator required parasite entry into the cell and sequestration into a mature phagolysosome (fig. S1A). Therefore, we investigated the role of the macrophage endosomal Toll-like receptors (TLRs) of the myeloid differentiation factor 88 (MyD88) (TLR7 and TLR9) and/or of the TIR domain-containing adapter-inducing interferon- β (TRIF)-dependent pathways (TLR3). Using macrophage functionally deficient for TLR3, 7, or 9, or for the adaptors MyD88 and TRIF, we found that the TLR3-TRIF- dependent pathway was essential for increased proinflammatory mediator expression after macrophage infection with *L.g.*M+ (Fig. 2 and fig. S1B). In addition, MyD88-dependent TLR7 activation within the macrophage was required for maximal secretion of the proinflammatory mediators after infection with M+ parasites (Fig. 2 and fig. S1B). In our system, TLR9 was not involved in *L.g.*M+-dependent macrophage responses, suggesting that recognition of *Leishmania*-derived DNA motifs by the host's TLR9 does not differ between the *Leishmania* strains (Fig. 2A).

In other murine models of infection, TLR3 ligation up-regulates proinflammatory mediators (TNF- α , IL-6, and chemokines) and type I interferons, resulting in organ damage (20–22). To confirm the role of TLR3 in the recognition of *L.g.*M+ parasites, we analyzed IFN- β expression. Infection with *L.g.*M+ induced significantly more IFN- β transcripts (31.14 ± 23.46) than *L.g.*M- clones (5.83 ± 4.27) after 6 hours by comparison with unstimulated macrophage controls. This increase was observed as early as 2 hours after infection (Fig.

2B). At the protein level, after macrophage infection, *L.g.M+* M5313-derived and h-MCL induced higher IFN- β secretion than *L.g.M-* parasites or h-CL parasites (Fig. 2C). Furthermore, this expression was TLR3-TRIF dependent, with the MyD88 signaling pathway augmenting secretion (Fig. 2C).

Endosomal TLRs recognize nucleic acid motifs, with TLR7 and TLR3 recognizing single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), respectively (23). Our experimental evidence suggested that nucleic acid-derived motifs were involved in the host macrophage response to infection with metastasizing *L.g.* parasites. We observed increased production of CCL5, TNF- α , and IL-6 in macrophages exposed to single-stranded ribonuclease (ssRNase)- and deoxyribonuclease (DNase)-treated nucleic acids derived from *L.g.M+* parasites, compared with *L.g.M-* and *L.major* LV39 (fig. S2). Although not statistically significant, these results suggested that the nucleic acid motif is resistant to ssRNase and DNase treatments and is likely to be dsRNA.

L.Viannia parasites, including *L.g.M5313(M+)* and *L.guyanensis* and *L.braziliensis* MCL human isolates, harbor the dsRNA *Leishmania RNA virus 1* (LRV1) (24–26). These viruses have a capsid coat protecting a 5.3-kb dsRNA genome (27). Metastasizing promastigotes had greater levels of LRV1 (*L.g.M+* or h-MCL-LRV^{high}) than nonmetastasizing promastigotes (*L.g.M-* or h-CL-LRV^{low}) as shown by the presence of a ~5.3-kb, DNase-insensitive, RNase III-sensitive band in agarose gels, and LRV1 quantification by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) (Fig. 3, A to C, and fig. S3A). We thus verified that macrophages treated with purified LRV1 dsRNA (fig. S3) induced a phenotype similar to that of macrophage infected with metastasizing parasites, and as shown by an increased expression of CXCL10, CCL5, TNF- α , IL-6, and IFN- β transcripts, this increase was TLR3 dependent (Fig. 3D). Because the *L.g.M5313 M+* and *M-* parasites were not isogenic, we performed new experiments with parasites derived from the WHO reference strain *L.g.M4147* that metastasizes in the hamster (28) and carries the LRV1-4 virus (29). Macrophage infection with *L.g.M4147-LRV^{high}* parasites produced significantly greater amounts of cytokines and chemokines than infection with its respective isogenic virus-free derivative *L.g.M4147LRV^{neg}*, in a TLR3-dependent manner (Fig. 3E and fig. S4) (30, 31). Similar parasite burdens were observed for all parasites infected into the wild-type and the TLR-, TRIF-, and MyD88-deficient macrophages (table S1).

A role for TLR3 and LRV1 in leishmaniasis development was analyzed *in vivo*, with TLR3^{-/-}, TLR7^{-/-}, and WT mice that were infected in the footpad. A significant decrease in footpad swelling, and diminished parasite burden, were observed in TLR3^{-/-} mice infected with *L.g.M+LRV^{high}* (M5313) or *L.g.M4147-LRV^{high}* parasites compared with wild-type mice (Fig. 4 and fig. S5). No consistent, significant decrease in disease pathology was observed between TLR3^{-/-} and wild-type mice infected with *L.g.M-LRV^{low}* (Lg17) or *L.g.M4147-LRV^{neg}* or between TLR7^{-/-} and wild-type infected mice with the different parasite isolates (Fig. 4 and Fig. S5). Further experimentation is required to elucidate the role of TLR7-dependent immune responses with respect to infection with LRV1-containing *Leishmania* parasites.

Our work showed that recognition of LRV1 within metastasizing *L.g.* parasites by the host promoted inflammation and subverted the immune response to promote parasite persistence (2, 3, 32). Because recognition of LRV1 within the metastasizing *L.g.* parasites arises early after infection, we hypothesize that LRV1 dsRNA is released from dead parasites, unable to survive within the host macrophage. These results could open the door to better diagnosis of risk for MCL disease and facilitate the development of new and more efficient treatment regimes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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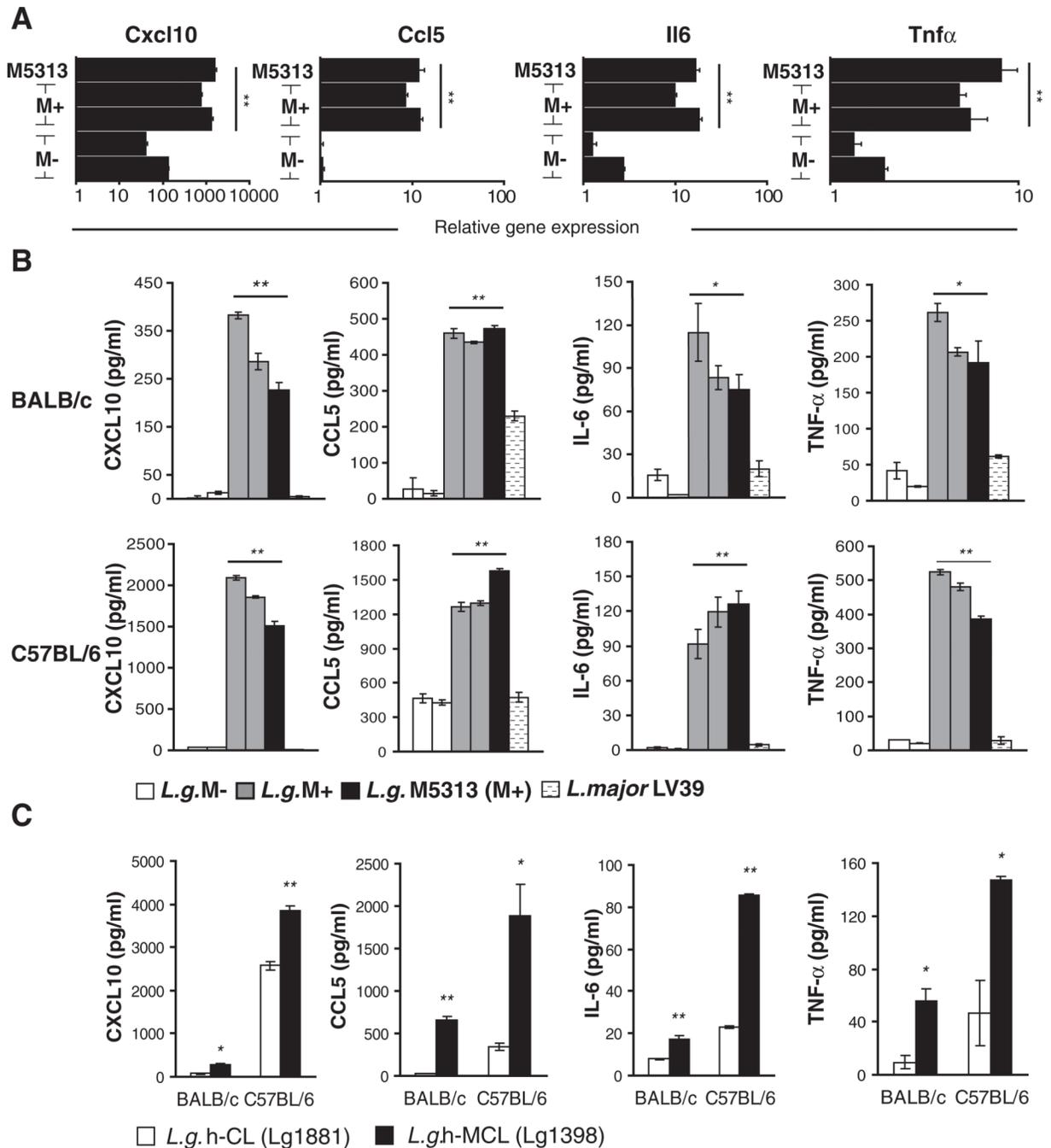


Fig. 1. Metastasizing *L.g.* parasites activate bone-marrow macrophages to elevate proinflammatory cytokine and chemokine levels. **(A)** Transcript and **(B)** and **(C)** secreted protein levels induced after C57BL/6 or BALB/c macrophage infection (ratio 1:10) with *Leishmania* parasites [two *L.g.*M⁻ clones (Lg03 and Lg17); two *L.g.*M⁺ clones (Lg13 and Lg21); *L.g.*M5313(M⁺); *L.g.* derived from h-MCL (*-L.g.*1398) or hCL (*L.g.*1881) lesions; and *L.major* LV39] for 6 hours. Results were confirmed in several independent experiments ($n > 3$), and data reflect mean \pm SD transcript or protein increase relative to unstimulated controls. Significance was determined at $*P \leq 0.05$, and $**P \leq 0.01$ for *L.g.*M⁺ or h-MCL versus *L.g.*M⁻, h-CL, and/or *L. major* LV39-stimulated macrophages.

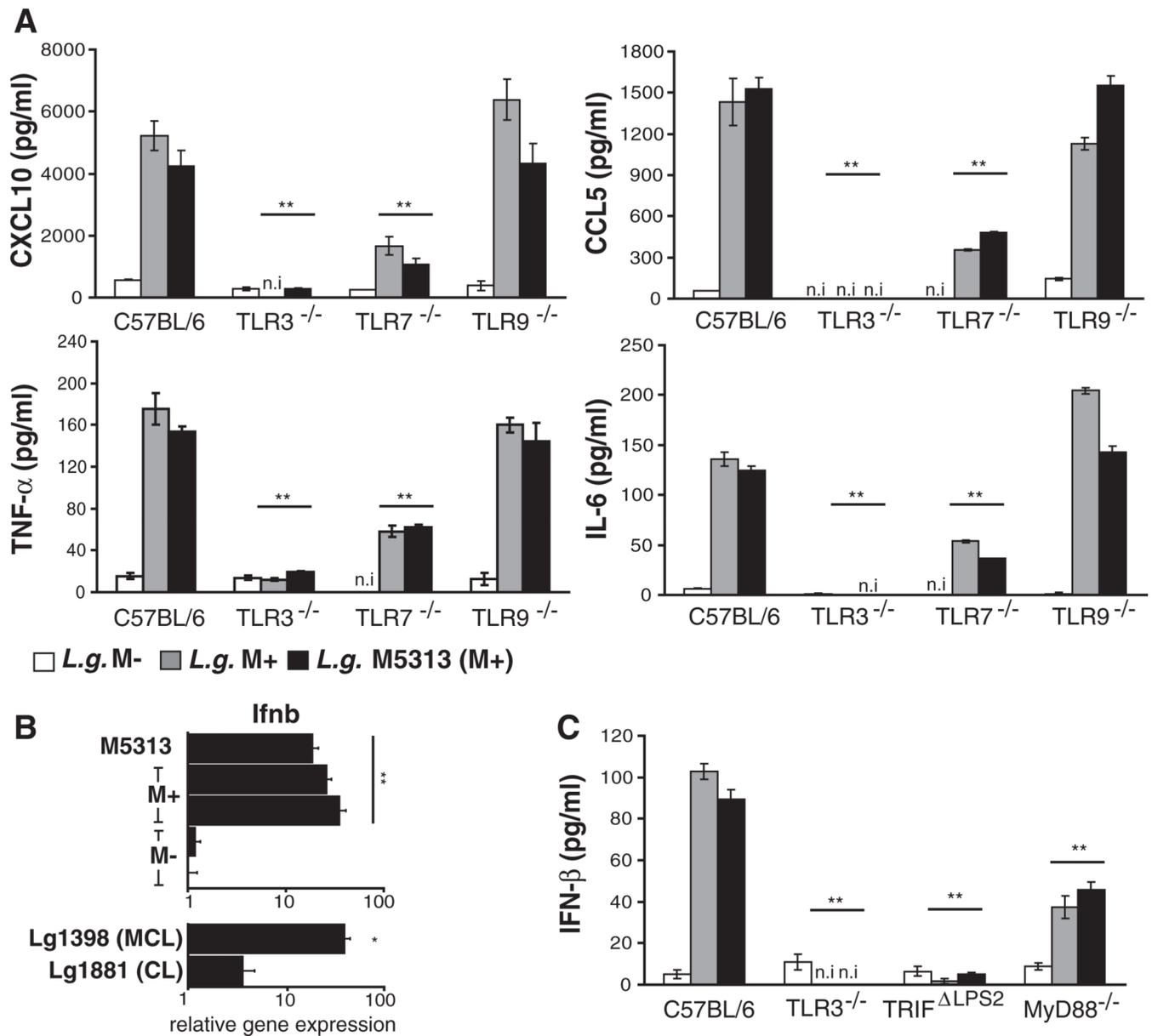


Fig. 2. *L.g. M+* or h-MCL parasite-dependent induction of IFN- β and proinflammatory mediators by macrophages uses TLR3 and TRIF. (**A** and **C**) Secreted protein and (**B**) transcript levels of cytokines and chemokines induced after infection of macrophages (ratio 1:10) with *Leishmania* parasites [two *L.g. M+* clones (Lg13 and Lg21), two *L.g. M-* clones (Lg03 and Lg17), and *L.g. M5313(M+)*] for 6 and 2 hours, respectively. Results were confirmed in several independent experiments ($n = 3$), and data reflect mean \pm SD transcript or protein increase relative to unstimulated controls of *L.g. M+* or *L.g. M-*. Significance was determined between C57BL/6 and deficient macrophages (A and C) or between *L.g. M+* or h-MCL and *L.g. M-* and h-CL parasites (B) at $*P \leq 0.05$ and $**P \leq 0.01$. n.i., not induced.

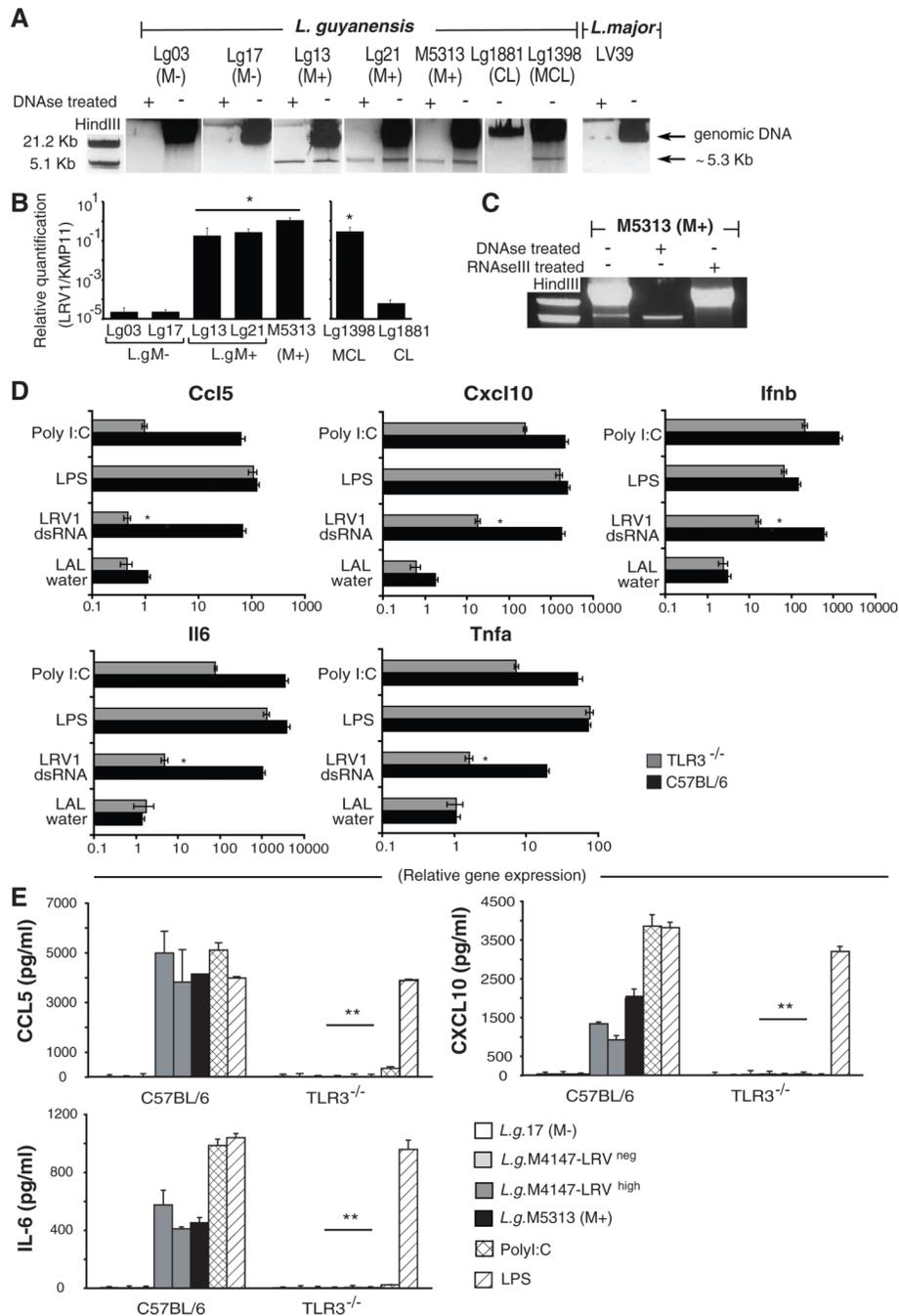
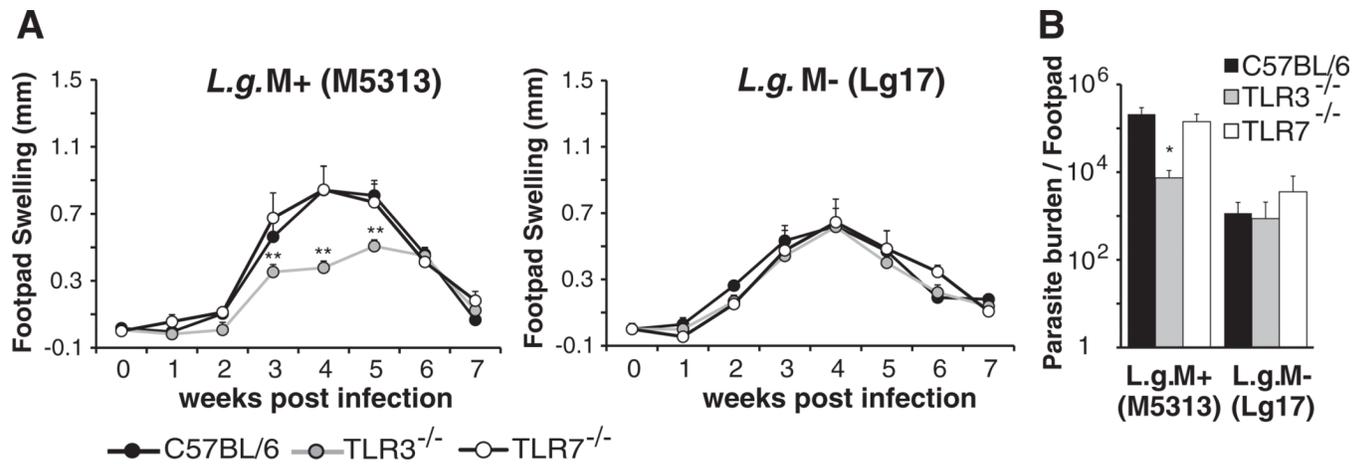


Fig. 3. High LRV1 burden within metastasizing *L.g.* promastigotes stimulates cytokine and chemokine production in macrophages via TLR3. (A) ssRNase-treated nucleic acids were DNase treated, and the 5.3-kb LRV1 dsRNA band visualized by gel electrophoresis. (B) LRV1 virus burden within *Leishmania* parasites was assessed by qRT-PCR with LRV1 and *Leishmania* Kmp11 gene primers; significance was determined between metastasizing (*L.g.*M+ and h-MCL) versus nonmetastasizing (*L.g.*M- and h-CL) parasites. (C) Nucleic acids from *L.g.*M5313(M+) promastigotes, pretreated with a ssRNA-specific RNase, were treated with DNase or with the dsRNA-specific RNase III and separated by gel electrophoresis with the marker Lambda-DNA-Eco RI + Hind III (HindIII). (D)

Macrophages were stimulated with purified LRV1 dsRNA (1 $\mu\text{g/ml}$) in endotoxin-free (LAL) water, poly(I:C) (1 $\mu\text{g/ml}$), or lipopolysaccharide (LPS, 100 ng/ml) for 4 hours. Transcript levels were assessed relative to unstimulated C57BL/6 macrophages by qRT-PCR. Results are expressed as mean \pm SD ($n=2$). (E) Protein abundance was quantified after infection of macrophages (ratio 1:10) with *L.g.M4147-LRV^{high}* (clones 2 and 3) or *L.g.M4147-LRV^{neg}* (clones 3 and 4) parasites after 6 hours. Controls: *L.g.M⁻* (Lg17), *L.g.M5313(M⁺)*, poly(I:C) (2 $\mu\text{g/ml}$), and LPS (100 ng/ml). Data reflect mean \pm SD of protein secretion relative to unstimulated controls ($n = 2$). Significance was determined at $*P \leq 0.05$ or $**P \leq 0.01$.

**Fig. 4.**

TLR3^{-/-} mice infected with *L.g.M+* parasites have decreased disease pathology when compared to wild-type C57BL/6. Footpads ($n \geq 4$) were infected with 3×10^6 parasites. **(A)** Footpad swelling was measured weekly and **(B)** parasite burden ($n=3$) was determined at 4 weeks after infection by qRT-PCR with *Leishmania* Kmp11 gene-specific primers. Representative data of two experiments, expressed as mean \pm SEM of all mice infected per group, with statistical significance at $*P \leq 0.05$ and $**P \leq 0.01$.



Supporting Online Material for

***Leishmania* RNA Virus Controls the Severity of Mucocutaneous Leishmaniasis**

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This PDF file includes:

Materials and Methods
Figs. S1 to S5
Table S1
References

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^{ALPS2} 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.M*5313(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°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.M*4147-LRV^{high}) and a virus free derivative (M4147/pX63-HYG-*L.g.M*4147-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 (5×10^7 photons/sec/ 1×10^6 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.M*4147 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 (BMM ϕ) for 5 days using complete DMEM supplemented with L929 conditioned media at 37⁰C. Differentiated BMM ϕ were coated onto microtiter plates and infected (1:10) with stationary phase *Leishmania* parasites for 2, 6 or 24hrs. BMM ϕ were also stimulated with LPS (Sigma-Aldrich[®]), Poly I:C (Invivogen), or CpG (Invivogen) at 200 or 100ng/ml, 8 or 1 μ g/ml and 5 μ M respectively or pretreated with Chloroquine (20 μ M), or Cytochalsin D (40 μ M), 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 BMM ϕ was calculated. Briefly, 750 BMM ϕ were counted in 3 randomly selected microscope fields of view and the average percentage infectivity and number of infected BMM ϕ was calculated.

DNA Microarray

Three biologically independent experiments were performed. For each experiment transcript levels were compared from RNA preparations of uninfected BMM ϕ 's or BMM ϕ '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 RNeasy 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 μ g 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 (2 μ l Alien spikes pool and 2 μ l 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 TNF α kits were purchased from R&D systems, IL6 (ebioscience) and IFN β (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 BMM ϕ 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'-CTGACTGGACGGGGGTAAT-3' and 5'-CAAACACTCCCTTACGC-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°C for 2 min and 95°C for 10 sec then followed by 40 cycles of 95°C for 15 sec, 60°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 10⁶ 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≤0.05, or p≤0.01 using the Student's *t* test.

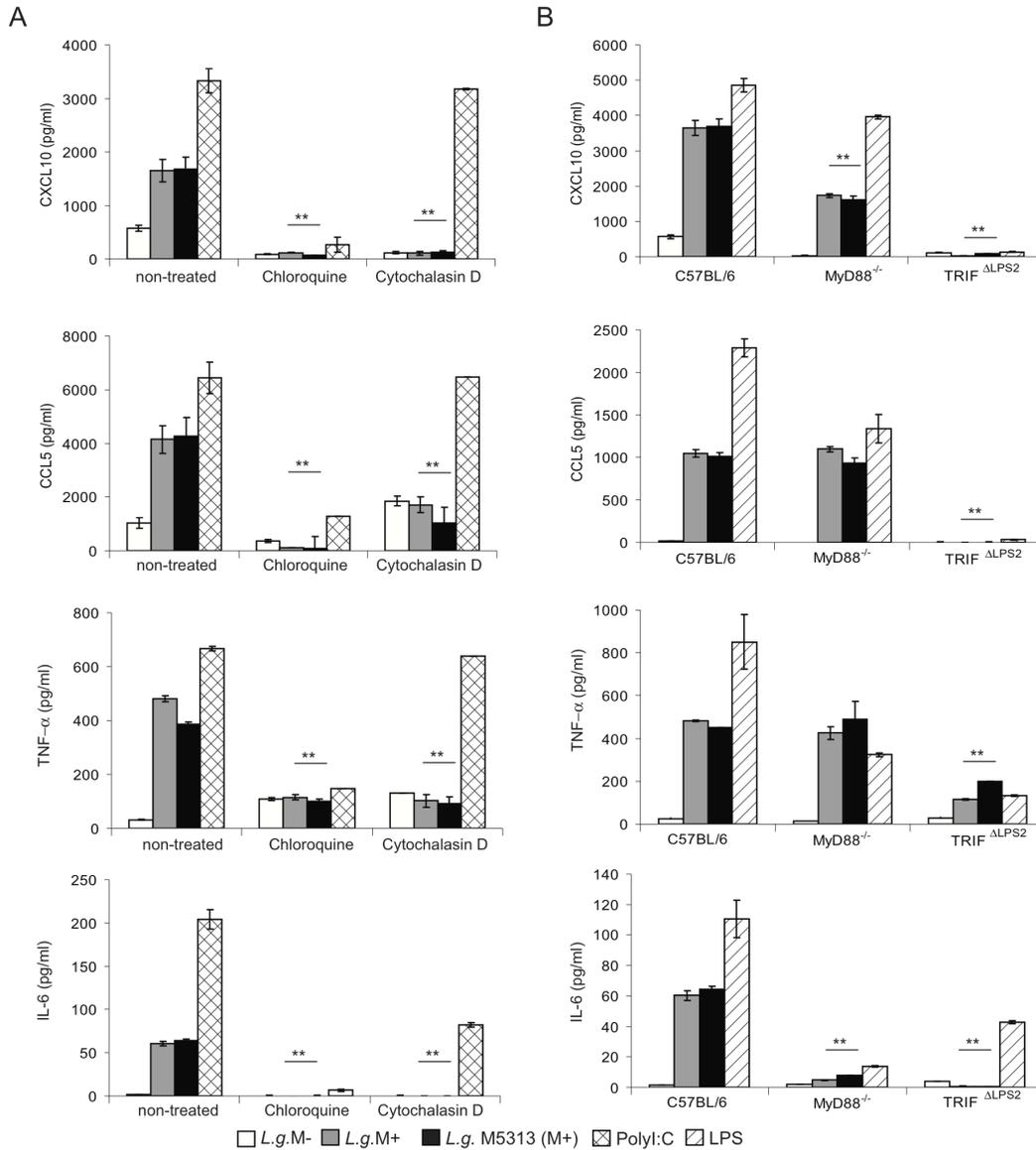


Fig. S1: Secretion of cytokines and chemokines by macrophages following *L.g.M+* infection requires internalization, and endosomal recognition of an RNA motif by the TRIF-dependant TLR signaling pathway. Secreted cytokine and chemokine proteins were determined in macrophages infected 1:10 with *L.g.* parasites for 6 hours. Protein secretion levels were compared in wild-type, untreated C57BL/6 macrophages versus those pretreated with either chloroquine (20 μ M, 2 hours), or cytochalasin D (40 μ M, 1 hour) (A) or compared, to MyD88^{-/-}, and TRIF^{ΔLPS2} macrophages (B). LPS and/or Poly I:C at 200ng/ml, and 8 μ g/ml respectively were included. Data reflects at least 3 independent experiments, with mean \pm SD protein concentration expressed above an unstimulated control, and statistical significance determined at (*) $p \leq 0.05$, and (**) $p \leq 0.01$.

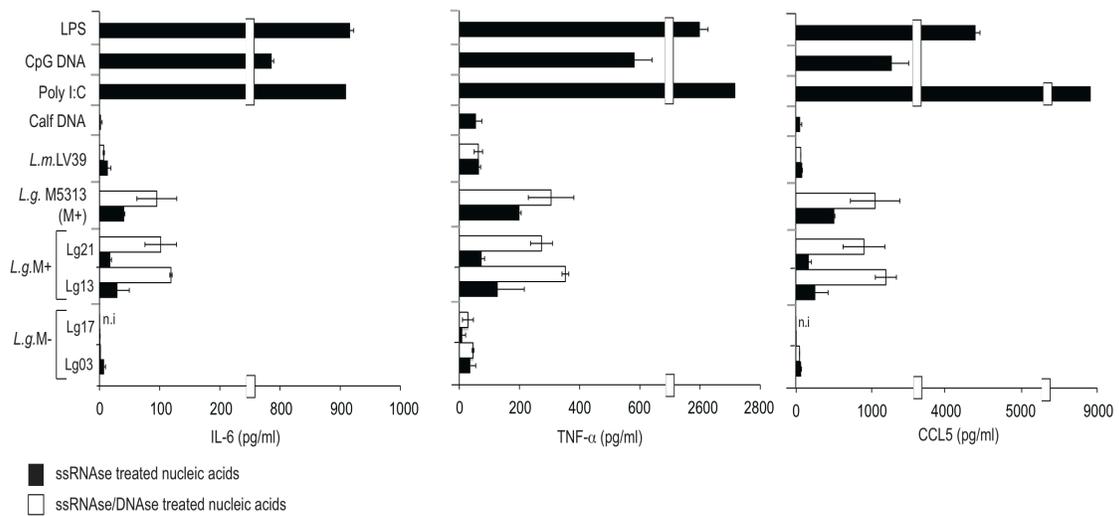


Fig. S2: Macrophages recognize a nucleic-acid-derived motif present in *L.g. M+* parasites. BALB/c macrophages were treated with ssRNase treated nucleic acids (5µg/ml) isolated by phenol/chloroform from *Leishmania* parasites or with ssRNase and DNase digested for 6 hours. Included within these experiments 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.

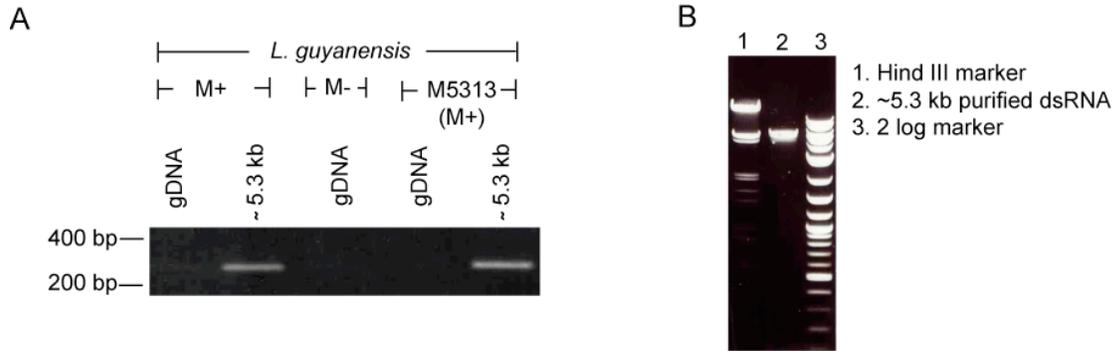


Fig. S3: Quality control and purification of LRV1 dsRNA from *L.g.M5313 (M+)*. **(A)** Genomic DNA (gDNA) and ~ 5.3kb LRV1 dsRNA bands were visualized, and extracted from a 1% agarose gel, following ssRNase treated total nucleic acids from stationary phase promastigotes of *L.g. M+* (Lg13), *L.g.M-* (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.

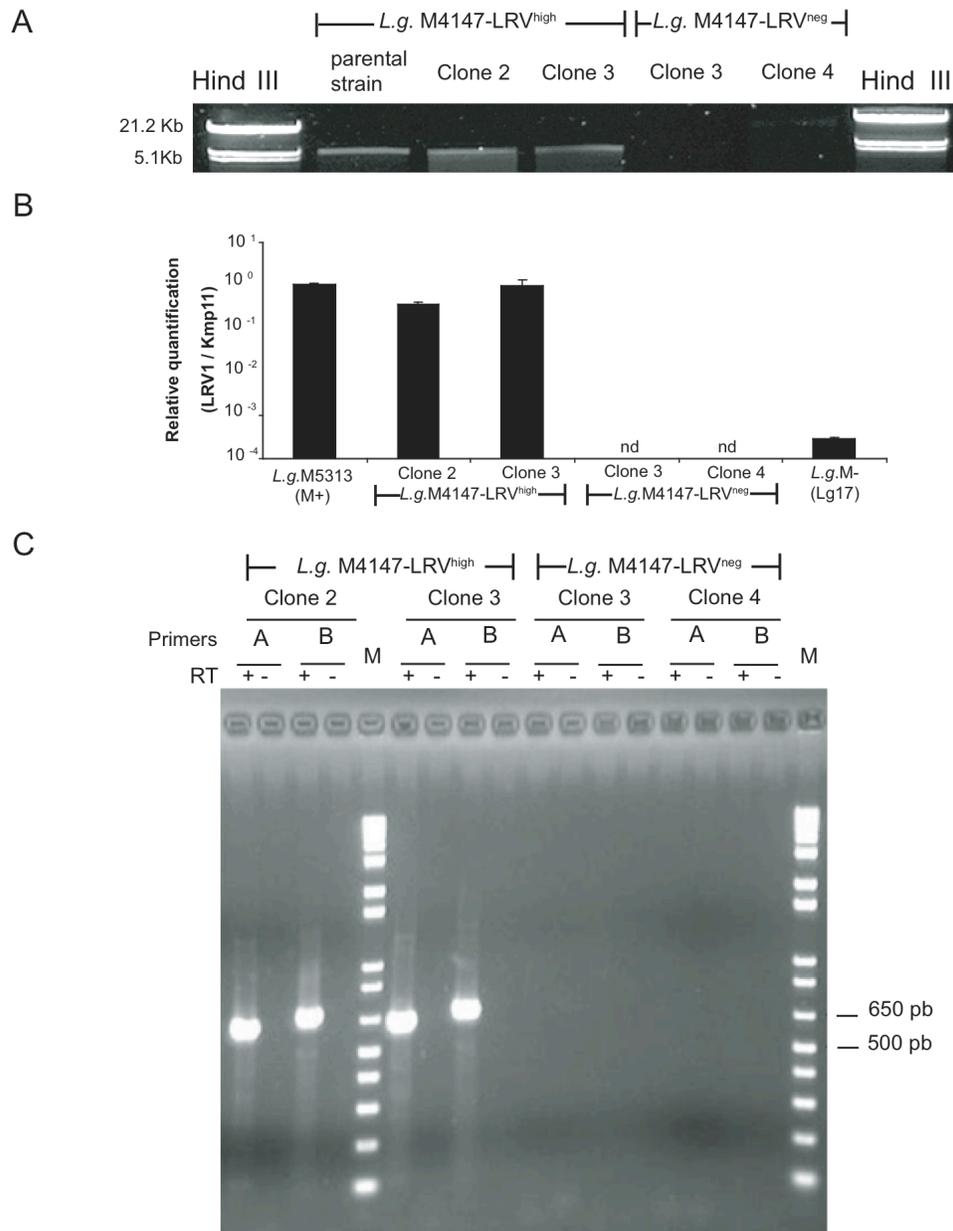


Fig. S4: Determination of the presence or the absence of LRV1-4 virus in *L.g.M4147* and in two independent clones of *L.g.M4147-LRV^{high}* and isogenic virus-free derivative *L.g.M4147-LRV^{neg}*. **(A)** ssRNAse treated nucleic acids were treated with DNase and the presence of the 5.3 kb LRV1 dsRNA 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.

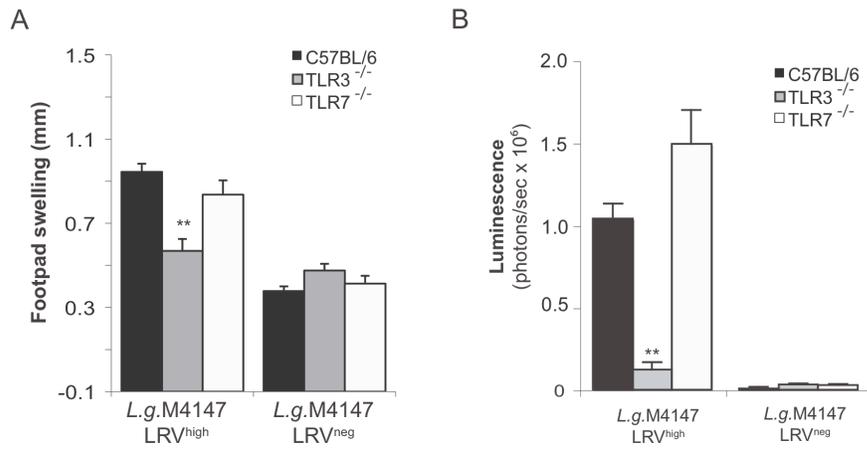


Fig. S5: TLR3^{-/-} mice infected with *L.g.M4147*-LRV^{high} parasites have less disease pathology when compared with WT C57BL/6. Footpads of mice (n ≥ 5) were infected with 3x10⁶ parasites. **(A)** Footpad swelling, and **(B)** parasite burden were determined at 4 weeks post infection. Parasite burden 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 \pm standard deviation of 3 different microscope fields of view.

		Infection rate (%)	No. parasites per infected macrophages
<i>Leishmania</i> parasites infected into C57BL/6 macrophages			
<i>L.g.M-</i>	Clone Lg03	88.04 \pm 5.03	5.90 \pm 2.39
	Clone Lg17	92.15 \pm 2.10	7.10 \pm 0.88
<i>L.g.M+</i>	Clone Lg13	93.83 \pm 4.60	7.63 \pm 3.06
	Clone Lg21	88.77 \pm 8.28	6.72 \pm 1.85
	M5313	94.93 \pm 4.30	10.24 \pm 6.22
<i>L.major</i>	LV39	93.67 \pm 3.94	6.51 \pm 1.89
<i>L.g.</i>	M4147LRV ^{high}	94.87 \pm 2.10	5.75 \pm 1.43
	M4147LRV ^{neg}	93.3 \pm 3.05	4.71 \pm 1.17
Macrophages infected with <i>L.g.M5313</i> (M+)			
C57BL/6 (Wildtype)		90.46 \pm 7.11	7.6 \pm 3.0
TLR3 ^{-/-}		88.66 \pm 1.44	5.6 \pm 2.5
TLR7 ^{-/-}		88.64 \pm 5.71	5.9 \pm 2.6
TLR9 ^{-/-}		89.49 \pm 6.97	8.1 \pm 3.0
TRIF ^{ΔLPS2}		91.44 \pm 2.95	6.3 \pm 1.7
Myd88 ^{-/-}		91.99 \pm 4.54	6.8 \pm 2.7

Supplementary material references

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