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## Neutrophil elastase promotes *Leishmania donovani* infection via interferon- $\beta$

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**ABSTRACT:** Visceral leishmaniasis is a deadly illness caused by *Leishmania donovani* that provokes liver and spleen inflammation and tissue destruction. In cutaneous leishmaniasis, the protein of *L. major*, named inhibitor of serine peptidases (ISP) 2, inactivates neutrophil elastase (NE) present at the macrophage surface, resulting in blockade of TLR4 activation, prevention of TNF- $\alpha$  and IFN- $\beta$  production, and parasite survival. We report poor intracellular growth of *L. donovani* in macrophages from knockout mice for NE (*ela*<sup>-/-</sup>), TLR4, or TLR2. NE and TLR4 colocalized with the parasite in the parasitophorous vacuole. Parasite load in the liver and spleen of *ela*<sup>-/-</sup> mice were reduced and accompanied by increased NO and decreased TGF- $\beta$  production. Expression of ISP2 was not detected in *L. donovani*, and a transgenic line constitutively expressing ISP2, displayed poor intracellular growth in macrophages and decreased burden in mice. Infected *ela*<sup>-/-</sup> macrophages displayed significantly lower IFN- $\beta$  mRNA than background mice macrophages, and the intracellular growth was fully restored by exogenous IFN- $\beta$ . We propose that *L. donovani* utilizes the host NE-TLR machinery to induce IFN- $\beta$  necessary for parasite survival and growth during early infection. Low or absent expression of parasite ISP2 in *L. donovani* is necessary to preserve the activation of the NE-TLR pathway.—Dias, B. T., Dias-Teixeira, K. L., Godinho, J. P., Faria, M. S., Calegari-Silva, T., Mukhtar, M. M., Lopes, U. G., Mottram, J. C., Lima, A. P. C. A. Neutrophil elastase promotes *Leishmania donovani* infection via interferon- $\beta$ . FASEB J. 33, 10794–10807 (2019). www.fasebj.org

**KEY WORDS:** toll · serine protease · inhibitor · ISP

The leishmaniasis comprise a spectrum of diseases that vary from self-healing cutaneous lesions to visceral infections that are caused by more than 20 species of *Leishmania*.

**ABBREVIATIONS:** Ag, antigen; BSA, bovine serum albumin; FCS, fetal calf serum; IRF, IFN regulatory transcription factor; IRAK, interleukin-1 receptor-associated kinase 1; ISP, inhibitor of serine peptidases; LdISP2, *Leishmania donovani* inhibitor of serine peptidase 2; L-NAME, N<sup>ω</sup>-nitro-L-arginine methyl ester hydrochlorid; MyD88, myeloid differentiation primary response 88; NE, neutrophil elastase; NEI, irreversible inhibitor of NE; qPCR, quantitative PCR; ROS, reactive oxygen species; poly:IC, polyinosinic-polycytidylic acid sodium salt; RPMI, Roswell Park Memorial Institute; TBS-T, Tris-buffered saline with 0.05% Tween 20; TRAF, TNF receptor-associated factor; TRIF, TIR-domain-containing adapter-inducing interferon  $\beta$ ; VL, visceral leishmaniasis; WT, wild type

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Visceral leishmaniasis (VL) is a severe and potentially fatal disease characterized by pathologies in the liver and spleen. It remains a major cause of mortality in many tropical countries, with an incidence of ~90,000 new cases per year and ~350 million people at constant risk of infection (1) in regions including Africa, Southeast Asia, southern Europe, eastern Mediterranean, and Latin America. VL is caused by the intracellular protozoa *Leishmania infantum* (syn. *L. chagasi*), which is prevalent in Latin America, or *L. donovani*, which is prevalent throughout and temperate regions of Africa and southern Asia (1). The parasite replicates in the phlebotomine vector as the flagellated promastigote form that subsequently differentiates into infective metacyclic promastigotes. Following the bite of infected sand flies, metacyclics are internalized by phagocytes and replicate as amastigotes in the parasitophorous vacuole of infected macrophages, maintaining chronic infection (2).

Both host genetic background and immunity and parasite-related determinants are likely crucial to define a viscerotropic phenotype and pathology (3). Comparison of the genomes of species that cause cutaneous leishmaniasis with different clinical manifestations [*i.e.*, *L. major*, *L. braziliensis*, *L. mexicana*, and the genome of the *L. donovani*

(4, 5)] revealed only 19 *L. donovani*-specific genes. Differential gene expression, rather than species-specific genes, could play a central role in the parasite phenotype and clinical outcome. Studies comparing a naturally attenuated strain of *L. donovani* from Sri Lanka that causes cutaneous infection in a population with increased virulence recovered after systematic passage through the viscera of mice revealed increased levels of proteins associated with stress antioxidant responses (6). In experimental infections, the immune response of the mammalian host contributes to promote or control the parasite burdens. As an example, agents of innate immunity such as TLRs have been implicated in parasite control: expression of TLR2 and TLR4 in the liver were associated with parasite burden in *L. infantum*-infected mice (7), and TLR4 was proposed to act in synergy with NK cells to control VL during immunotherapy (8). On the other hand, studies in TLR knockout mice suggested that TLR2 and TLR4 play antagonizing roles in the promotion *vs.* control of *L. donovani* burden, respectively (9), whereas engagement of TLR2 and of the mannose receptor was recently suggested to slow down phagosome maturation in infected macrophages (10). In addition to microbe-derived TLR ligands, host-derived molecules such as  $\alpha$ -synuclein (11), heparan sulfate (12), fibrinogen (13), fibronectin (14), and the serine peptidase neutrophil elastase (NE) can also activate TLRs to trigger innate response mechanisms (15). Of relevance to *Leishmania* infection, NE was implicated in *L. major* killing in macrophages through a TLR4-dependent mechanism (16, 17).

We have previously described that *L. major* has 3 single genes [inhibitor of serine peptidases (*ISP*) 1, *ISP2*, and *ISP3*] that share similarity with bacterial ecotin, which encodes an inhibitory protein of serine peptidases (18). Transgenic *L. major* knockout for *ISP2* and *ISP3* is more susceptible to killing by macrophages and displays delayed intracellular growth related to superoxide production triggered by an NE-TLR4-dependent mechanism (17). Such responses also require TLR2, resulting in the activation of protein kinase R and the production of TNF- $\alpha$  and IFN- $\beta$ , which contribute to parasite killing (19). *ISP2* knockout parasites were likewise unable to sustain prolonged infections in C57BL6 mice, which was paralleled with increased infiltration of NOS2-expressing monocytes at the lesion site and increased signs of inflammation (20). Those observations helped to define *ISP2* as a virulence factor required for successful infection by *L. major* (21, 22), but the potential contribution of *ISP2* in modulating NE activity and infection in VL has not been investigated. mRNA to *ISP2* was detected in *L. donovani* (23), and studies *in vitro* using recombinant *ISP2* from this species [*L. donovani* inhibitor of serine peptidase 2 (LdISP2)] showed that it can inhibit trypsin and NE (24, 25). Recombinant *ISP2* also inhibited serine peptidases of the sand fly midgut *in vitro*, and transgenic lines knocked down to *ISP2* were more susceptible to sand fly peptidases and less fit to survive in the midgut (23). Other studies applying treatment of infected RAW cells with recombinant LdISP2 showed slight reduction of infection and of NO production (25). Recombinant LdISP2 was recently shown to inhibit the complement-related serine peptidases and C3c

and C5c generation (26). However, the *ISP2* gene is truncated in the genome of the *L. donovani* reference strain BPK282A1, suggesting that some *L. donovani* strains might not express a functional *ISP2* inhibitor, which could influence the control of serine peptidase activity during infection. We asked if the engagement of the NE-TLR signaling platforms played a role in visceral infection.

We show that *L. donovani* depends on NE activity to establish infection in macrophages and to develop parasite burden in the liver. Furthermore, the molecular mechanism associated with NE dependency requires TLR4 and IFN- $\beta$ , and IFN- $\beta$  production is significantly reduced upon expression of *ISP2* in transgenic *L. donovani*, negatively affecting parasite fitness.

## MATERIALS AND METHODS

### Parasites and transgenic mice

*L. donovani* strain from the Sudan (MW897) was grown as promastigotes in hemoflagellate modified Eagle's medium (designated HOMEM medium) supplemented with 10% heat-inactivated fetal calf serum (FCS) at 25°C. Parasites were passed through CB57BL6 mice for 20 d and recovered from the spleen for culture in HOMEM-FCS. The cultures were diluted (passed) weekly at  $2 \times 10^6$ /ml and kept for up to 5 passages. Extracellular amastigotes were generated by culturing *L. donovani* stationary-phase promastigotes in Schneider's *Drosophila* medium (Thermo Fisher Scientific, Waltham, MA, USA), pH 5.4, supplemented with FCS 20% at 37°C for 6 d. Viability of the amastigotes was confirmed through the observation of parasite growth as amastigotes by daily counting in Neubauer chamber. NE knockout mice (*ela2*<sup>-/-</sup>) (27) were kindly donated by Dr. Bernhard Ryffel [Centre National de la Recherche Scientifique (CNRS), Orleans, France], and the colonies were kept at Laboratório de Animais Transgênicos (LAT) at Universidade Federal do Rio de Janeiro (UFRJ; Rio de Janeiro, Brazil). *Tlr2*<sup>-/-</sup> and *tlr4*<sup>-/-</sup> mice were donated by Dr. Marcelo Bozza (UFRJ) for each experiment. All mice were handled according to approved ethics committee [Comissão de Ética no Uso de Animais (CEUA) 034/15-UFRJ].

### Generation of *L. donovani* transgenic lines

A fragment containing the *L. major* *ISP2* open reading frame (477 bp) was generated by PCR using as primers OL1812 and cloned into pGL1177 downstream of the 5'UTR of the rRNA gene, as previously described by Eschenlauer *et al.* (18). The construct was digested with PacI and PmeI and purified from agarose gels prior to transfection. In total,  $5 \times 10^7$  midlog-phase promastigotes forms of *L. donovani* were electroporated with 10  $\mu$ g of the linearized integration cassettes using the Amaxa electroporator (Lonza Corp., Portsmouth, NH, USA). The transfectants were cloned by limiting dilution and selected with 50  $\mu$ g/ml puromycin. Genomic DNA from the resistant clones was isolated to check for the correct integration of the constructions by PCR.

### Western blotting

In total,  $2 \times 10^7$  stationary-phase promastigotes of *L. major* or *L. donovani* or  $2 \times 10^7$  axenic amastigotes of *L. donovani* were collected, washed in PBS, resuspended in 50  $\mu$ l of SDS-PAGE sample buffer containing 2-ME, and boiled for 5 min. The soluble fractions of cell lysates were separated by 14% SDS-PAGE, and the proteins were transferred onto a PVDF membrane. The membrane was blocked

in Tris-buffered saline with 0.05% Tween 20 (TBS-T) with 9% w/v nonfat dry milk for 1 h at room temperature. Membranes were washed 3 times for 5 min each with TBS-T. Sheep antiserum raised against recombinant ISP2 (18) was diluted 1:300 in TBS-T and incubated for 2 h. After 3 washes, secondary anti-sheep antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, USA) were used at a 1:10,000 dilution and incubated for 2 h. Antibodies to  $\alpha$ -tubulin (MilliporeSigma, Burlington, MA, USA) at 1:4000 dilution were used for loading control.

## Macrophage infection assays

Peritoneal macrophages from C57BL/6, *ela2*<sup>-/-</sup>, *tlr2*<sup>-/-</sup>, and *tlr4*<sup>-/-</sup> mice were elicited by injection of 1% thioglycolate sterile solution in the peritoneal cavity. After 3 d, the cells were collected upon injection of 5 ml of ice-cold Roswell Park Memorial Institute (RPMI) 1640 medium (MilliporeSigma) in the peritoneal cavity. The cells were washed and plated onto glass coverslips at  $4 \times 10^5$  cells per well in a 24-well tissue culture plate and cultivated overnight at 37°C in RPMI supplemented with 10% of FCS (Cultilab, Campinas, Brazil). After 24 h, the monolayers were washed with HBSS, and the adherent cells were infected with late-stage promastigotes (culture density of  $3 \times 10^7$ /ml) of *L. donovani* at a 5:1 parasite:cell ratio in RPMI and supplemented with 0.1% bovine serum albumin (BSA; MilliporeSigma) at 37°C for 3 h, followed by washing, fixation with 70% methanol, and Giemsa staining. In all experiments, parasites that recovered from infected mice and were kept for 3–5 weekly passages in culture were used. For survival assays, after the 3 h infection, the cultures were washed 3 times with HBSS for removal of extracellular parasites and further cultured for 24 or 72 h in RPMI 10% FCS before fixation and staining. The number of intracellular parasites was determined by counting at least 100 cells per replicate under the light microscope at  $\times 100$  oil immersion objective. Where indicated in the figure legends, the irreversible inhibitor of NE (NEI), *O*-methoxy-succinyl-alanyl-alanyl-prolyl-alanyl-chloro-methylketone (10  $\mu$ M; Calbiochem, San Diego, CA, USA), was added to the macrophages 5 min before addition of the parasites. In the experiments of receptor neutralization, 10  $\mu$ g/ml anti-mouse TLR4 neutralizing antibodies (CD284/MD2 complex clone MTS510; Thermo Fisher Scientific) or 10  $\mu$ g/ml IgG (IgG2aK control clone eBR2a) were incubated for 30 min with macrophages in RPMI-FCS, and the monolayers were washed twice before infection with the parasites at 37°C in RPMI-BSA. Where indicated in the figures, after 3 h of infection followed by washing, 200 ng/ml NE (Calbiochem) was added, and the cultures were cultivated for 72 h in RPMI-FCS. One thousand units per milliliter IFN- $\beta$  (BioLegend, San Diego, CA, USA) or different concentrations of IFN- $\alpha$  (Thermo Fisher Scientific) were added to cultures that had been previously infected for 3 h and washed for removal of extracellular parasites and cultivated for 72 h in RPMI-FCS. Where appropriate, 50  $\mu$ M EUK134 (Calbiochem) or 1 mM of NW-nitro-L-arginine methyl ester hydrochloride (L-Name; MilliporeSigma) were preincubated with macrophages and washed before infection. Twenty-five micrograms per milliliter polyinosinic-polycytidylic acid sodium salt (poly:IC) was added to cultures after 3 h of infection and incubated for the indicated times.

## Immunofluorescence

Thioglycolate elicited peritoneal macrophages from C57BL/6 mice plated onto coverslips in 24-well tissue culture plates cultured overnight in RPMI-FCS. After 24 h, monolayers were washed 3 times, and adherent macrophages were infected with *L. donovani* or *L. donovani*:ISP2 in RPMI supplemented with 0.1% BSA for 3 h at 37°C. The cultures were washed and fixed with 1% paraformaldehyde for 15 min at 37°C, followed by washes with

PBS or washed and cultivated in RPMI-FCS for 72 h before fixation. After the fixation step, the cells were permeabilized with 0.1% Triton X-100 for 5 min at room temperature, washed with PBS, incubated with 0.1 M glycine for 20 min at room temperature, and washed and blocked in PBS containing 1% BSA for 1 h. The cells were incubated with rabbit anti-NE antibodies at 1:200 dilution (ab68672; Abcam, Cambridge, MA, USA) for 2 h. After, the coverslips were extensively washed with PBS and incubated with cyanine 3-conjugated anti-rabbit IgG (1:1,000 dilution) for 1 h. After washing, the cells were incubated with anti-TLR4 antibodies at 1:100 dilution (ab13556; Abcam) for 2 h. After, the cells were extensively washed with PBS and incubated with 488-conjugated anti-rabbit IgG at 1:1,000 dilution for 1 h. Coverslips were treated with DAPI for 5 min. The slides were mounted in Dabco on coverslips. Samples were analyzed by Zeiss LS480 confocal microscope (Carl Zeiss, Oberkochen, Germany) under a  $\times 60$  oil immersion objective.

## In vivo infections

In total,  $3 \times 10^7$  stationary-phase promastigotes in PBS were injected in the retro-orbital cavity of C57BL/6 mice or *ela2*<sup>-/-</sup> mice (6 mice per group). After 7 d, the mice were euthanized, and the spleen and liver were collected. The liver was ruptured to homogeneity with the back of a syringe through a nylon membrane in HOMEEM medium (Thermo Fisher Scientific) supplemented with 10% FCS. One hundred microliters of the cell homogenates were submitted to 24-fold serial dilutions in 96-well plates and cultivated for 7 d in 27°C for parasite burden analysis; the highest dilution well was used to estimate the number of parasites multiplied by the dilution factors. The spleens were macerated and diluted in serial 10-fold dilutions (1:10, 1:100, 1:1000). The cultures were maintained in HOMEEM medium supplemented with 10% FCS for 7 d for parasite growth before estimation of burdens.

## ELISA and nitrite concentration

In total,  $3 \times 10^7$  stationary-phase promastigotes were injected in the retro-orbital cavity of C57BL/6 mice or *ela2*<sup>-/-</sup> mice (6 mice/group) in PBS. After 7 d, the mice were euthanized, and the organs (spleen and liver) were macerated as previously described. The cells were centrifuged and resuspended in ammonium-chloride-potassium solution for 30 s followed by addition of PBS. The cells were collected by centrifugation at 200 *g* for 5 min, and PBS wash steps were repeated 2 additional times. The cells were centrifuged and resuspended in RPMI-FCS. Subsequently, the cells were plated in 48-well plates and cultivated for 48 h before collection of supernatants. Where indicated, parasite total antigen (Ag) was obtained by repeated cycles freeze-thaw of  $1 \times 10^7$  *L. donovani* promastigotes. Cytokine concentrations in supernatants were determined by ELISA (BD Biosciences, San Jose, CA, USA). Nitrite was measured with 50  $\mu$ l of culture supernatants by the Griess method.

## Quantitative PCR assays

Thioglycolate-recruited peritoneal macrophages from C57BL/6 or *ela2*<sup>-/-</sup> mice were infected with stationary-phase promastigotes at a 10:1 parasite:cell ratio in RPMI-BSA at 37°C for 2 or 6 h. The cells were washed for removal of noninternalized parasites. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Germantown, MD, USA), and cDNA was synthesized using 1  $\mu$ g of RNA and the Imprimar Kit (Promega, Madison, WI, USA). Real-time quantitative PCR (qPCR) assays of first-strand cDNA were performed with Step One (Thermo Fisher Scientific) and Sybr Green (Promega). The expression ratios were computed *via* the  $\Delta\Delta C_t$  method. The primers used were as

follows: glyceraldehyde 3-phosphate dehydrogenase (sense: 5'-TGCACCACCACCTGCTTAGC-3', antisense: 5'-GGCATGG-  
ACTGTGGTCATGAG-3'); IFN- $\beta$  (sense: 5'-TCCAAGAAAAG-  
GACGAACATTCG-3', antisense: 5'-TGAGGACATCTCCCAC-  
GTCAA-3'); IFN- $\alpha$  (sense: 5'-CTGGCTGTGARRAMATACTCC-  
3', antisense: 5'-TTCTGCTCTGACMACCTCC-3').

## Statistical analyses

Statistical analyses were performed using Prism 7.0 (GraphPad Software, La Jolla, CA, USA). The data were analyzed by 1-way ANOVA using the Bonferroni posttest at a significance level of 5%. The data were analyzed by 2-way ANOVA using the Bonferroni posttest at a significance level of 5%. The data were analyzed by multiple Student's *t* tests.

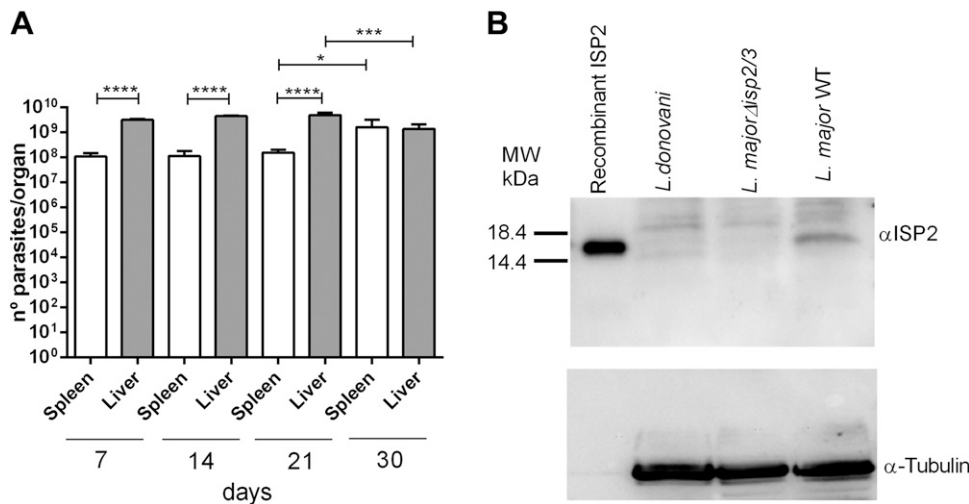
## RESULTS

To address the role of NE and its potential modulation by ISP2 in infections with viscerotropic *Leishmania*, we used an *L. donovani* strain from the Sudan (MW897). First, we evaluated the ability of this strain to cause VL in experimental murine infections. To this end, we injected stationary-phase promastigotes into mice and measured parasite burdens in the viscera of these animals. We observed parasitemia in both liver and spleen 7 d postinfection (Fig. 1A). The burdens at the liver were 10-fold higher than in the spleen up to d 21. At d 30, the parasite burden of the liver decreased, whereas in the spleen, the parasite burden increased 10-fold. Those observations prompted us to use this model to address the potential role of the NE-TLR axis underlying infection and its regulation by parasite ISP2.

Considering that in the *L. donovani* BPK282A1 (reference strain: TrypDB) genome the ISP2 gene (LdBPK\_150530.1) is a pseudogene (truncated), we verified the ISP2 gene in the *L. donovani* MW897 strain. The ISP2 gene was cloned and sequenced and found to be intact (Supplemental Fig. S1). The overall predicted amino acid sequence similarity of *L. donovani* MW897 ISP2 to *L. major* ISP2 was ~91%. Next, we used pAb raised against recombinant

ISP2 (18) to check ISP2 expression at the protein level in *L. donovani* MW897 late-stage promastigotes by Western blot. We did not detect ISP2 in the lysates of *L. donovani* MW897, although it was readily observed in wild-type (WT) *L. major* and absent in ISP2 knockout *L. major* ( $\Delta isp2/3$ ) (Fig. 1B). ISP2 could be either not expressed or expressed below the detection limit by Western blotting in *L. donovani*. Lack of ISP2 expression supports the hypothesis that, in contrast to what has been observed in *L. major* infections (17, 19), the NE-TLR4 signaling axis is active in *L. donovani* infections.

We first evaluated the role of NE in the infection of peritoneal macrophages of C57BL/6 mice with *L. donovani* MW897 (Fig. 2). Most of the parasites that were phagocytosed after 3 h of interaction survived inside the macrophages for up to 24 h (Fig. 2A). However, in the presence of 10  $\mu$ M, an NEI (*O*-methoxy-succinyl-alanyl-alanyl-prolyl-alanyl-chloro-methylketone), which is specific for NE and proteinase 3, only ~50% of the parasites internalized after 3 h of interaction survived inside the macrophages for 24 h (Fig. 2A), suggesting that NE activity is important for parasite survival. We did not observe any effect of NEI in the growth of promastigotes in culture (unpublished results). Similar data were obtained when macrophages from NE knockout mice (*ela2*<sup>-/-</sup>) were infected (Fig. 2B), confirming that approximately half of the intracellular parasites were eliminated when NE is not present. Subsequently, we followed the infection up to 72 h to evaluate the contribution of NE to the growth of intracellular amastigotes. We observed comparable parasite reduction in 24 h and a modest increase in parasite numbers up to 48 h, followed by a significant increase at 72 h postinfection in WT macrophages (Fig. 2C). However, there were 3-fold fewer parasites in *ela2*<sup>-/-</sup> macrophages at 72 h as compared with macrophages of WT mice (Fig. 2C), suggesting that NE is important for the establishment of *L. donovani* infections in murine macrophages. Next, we added exogenous NE to infected macrophages to verify whether the lack of NE was solely accounting for the low parasite burdens observed in *ela2*<sup>-/-</sup> macrophages at 72 h (Fig. 2D). Exogenous NE did not significantly affect the number of intracellular parasites in

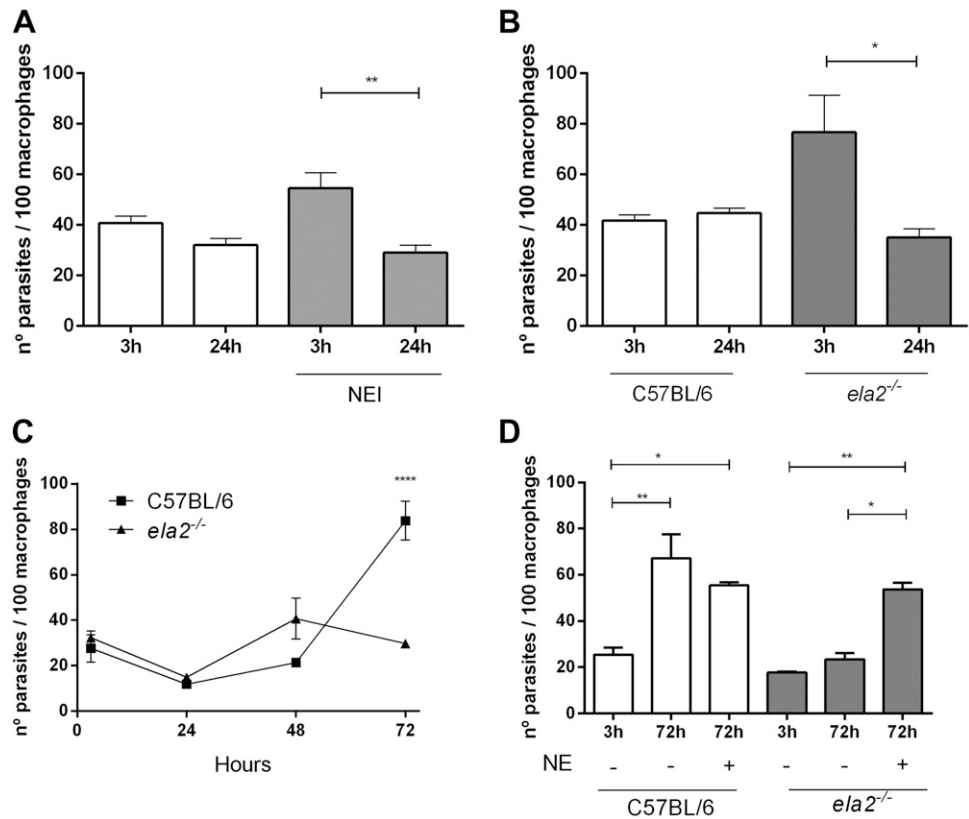


**Figure 1.** Course of *L. donovani* infection in C57BL/6 mice. A) Late stage, promastigotes of *L. donovani* MW897 ( $3 \times 10^7$ ) were washed in PBS and injected in C57BL/6 mice ( $n = 6$ ); at the indicated time points postinfection, parasite burdens were assessed by limiting dilution. The experiment was performed 3 independent times (6 animals/group). The figure shows 1 representative experiment. \* $P < 0.05$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . B) Expression of ISP2 is not detected in *L. donovani* MW897. Late-stage promastigotes ( $2 \times 10^7$ ) were washed twice in PBS and lysed in SDS-

PAGE sample buffer in reducing conditions, and ISP2 expression was assessed by Western blot using antiserum raised against recombinant *L. major* ISP2 (18). *L. major*  $\Delta isp2/3$  is a knockout line for the *ISP2* and *ISP3* genes and was used as a negative control. Antibodies to tubulin were used as loading control.

**Figure 2.** A, B) NE is required for the survival and intracellular growth of *L. donovani* in macrophages. Peritoneal thioglycolate-recruited macrophages from C57BL/6 mice (A) or from NE knockout mice (*ela2*<sup>-/-</sup>) (B) were cultivated on glass coverslips overnight in RPMI-FCS and washed and infected with late-stage promastigotes of *L. donovani* at a 5:1 parasite:macrophage ratio for 3 h at 37°C in RPMI supplemented with 0.1% BSA. C) The monolayers were washed 3 times to remove extracellular parasites, fixed with methanol and Giemsa stained (3 h), or further incubated for 24 h (A, B), 48 h, or 72 h in RPMI-FCS at 37°C before fixation and staining. The number of intracellular parasites was determined under the light microscope. Where indicated, the irreversible NEI OMeSuc-Ala-Ala-Pro-Val-CMK was added to the cultures at 10 μM final concentration immediately before addition of the parasites and kept

for the 3 h infection. D) Macrophages were infected as previously described for 3 h, and the monolayers were washed for removal of extracellular parasites and fixed (3 h) or cultured in RPMI-FCS for 72 h before fixation and staining. Where indicated (+), active purified human NE was added at 200 ng/ml final concentration to the cultures after the 3 h infection and remained for the 72 h. The experiments were performed in triplicate and repeated at least 3 independent times. The graphs show 1 representative experiment in triplicate. The graphs show means ± SD; statistical significance was assessed using 1-way ANOVA and the Bonferroni posttest. \**P* < 0.05, \*\**P* < 0.01, \*\*\*\**P* < 0.0001.



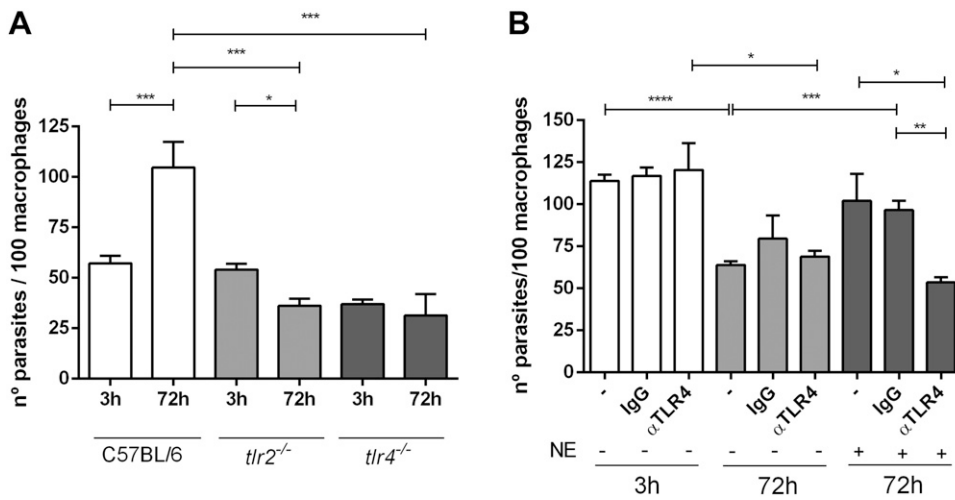
C57BL/6 macrophages in 72 h, whereas it restored amastigote numbers in *ela2*<sup>-/-</sup> macrophages similar to that of WT macrophage levels (Fig. 2D).

In macrophage infections with *L. major*, we and others have previously shown that NE exerts its leishmanicidal effect through TLR4 (16, 17). TLR2 is also implicated in the NE-dependent control of *L. major* infection in macrophages *in vitro* (19). Because we observed that lack of NE prevented the intracellular development of *L. donovani*, we tested whether the absence of TLRs would have the same effect (Fig. 3). In C57BL/6 macrophages, the number of intracellular parasites increased by 2-fold between 3 and 72 h (Fig. 3A, white bars). However, whereas macrophages from TLR2 knockout mice (*tlr2*<sup>-/-</sup>) internalized *L. donovani* to the same extent as WT macrophages in 3 h, we did not observe an increase in parasite numbers in the following 72 h in the *tlr2*<sup>-/-</sup> macrophages (Fig. 3A, gray bars), suggesting that TLR2 is required for parasite intracellular growth. The same phenotype was observed in macrophages from TLR4 knockout mice (*tlr4*<sup>-/-</sup>), in which the numbers of intracellular parasites 72 h post-infection were similar to those after uptake at 3 h and also significantly lower as compared with those in WT macrophages at 72 h (Fig. 3A, dark gray bars). To address if NE exerts its beneficial effect for the development of intracellular *L. donovani* through the NE-TLR4 axis, we tested whether the restoration of parasite growth in *ela2*<sup>-/-</sup> macrophages required TLR4. To that end, TLR4 was blocked by neutralizing

antibodies prior to the addition of exogenous NE (Fig. 3B). As expected, neutralizing antibodies to TLR4 did not affect parasite uptake in 3 h, and we observed a decrease in parasite numbers at 72 h in macrophages untreated or treated with anti-TLR4 or with control IgG (Fig. 3B). Addition of exogenous NE restored parasite growth to WT levels in 72 h, which was likewise observed in control IgG-treated macrophages. In contrast, exogenously added NE was unable to restore parasite growth in macrophages that had been pretreated with anti-TLR4, indicating that optimal intracellular growth of *L. donovani* depends on the NE-TLR4 axis.

We next analyzed the potential contribution of NE to parasite survival and growth in experimental infections in mice. At d 7 of infection, C57BL/6 mice displayed significant parasite burden in the liver, whereas in *ela2*<sup>-/-</sup> mice, liver parasite burdens were reduced by 5-fold comparatively (Fig. 4A). The burdens in the spleen were about 100-fold lower than in the liver at this time point and, likewise, were 5-fold lower in *ela2*<sup>-/-</sup> mice as compared with that of C57BL/6 mice (Fig. 4B), showing that NE also favors infection by *L. donovani* *in vivo*.

In *L. donovani* infections, T cell-derived TGF-β and IL-10 have been associated with the suppression of anti-leishmanicidal activity, thus promoting the establishment of the infection (28, 29). The differences in parasite burdens observed in the *ela2*<sup>-/-</sup> mice prompted us to determine the levels of selected cytokines. We detected decreased



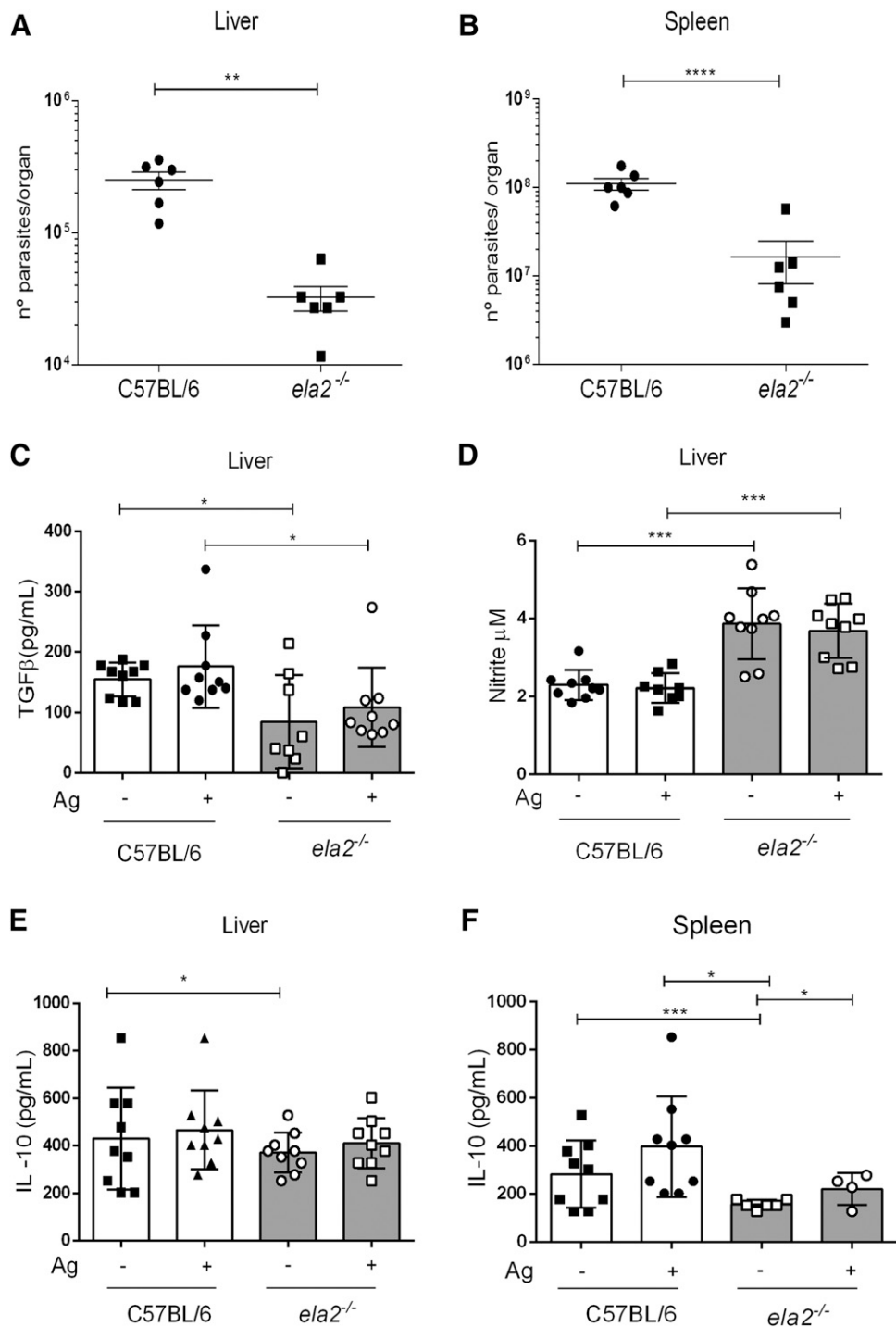
**Figure 3.** TLR4 and TLR2 are required for development of *L. donovani* in macrophages, and NE acts through TLR4. Peritoneal thioglycolate-recruited macrophages from C57BL/6 mice, TLR2 knockout mice (*tlr2*<sup>-/-</sup>), or TLR4 knockout mice (*tlr4*<sup>-/-</sup>) (A) or from NE knockout mice (*ela2*<sup>-/-</sup>) (B) were cultivated on glass coverslips overnight in RPMI-FCS and washed and infected with late-stage promastigotes of *L. donovani* at a 5:1 parasite:macrophage ratio for 3 h at 37°C in RPMI supplemented with 0.1% BSA. The monolayers were washed 3 times to remove extracellular parasites, fixed with methanol and Giemsa stained (3 h), or further cultivated for 72 h in RPMI-FCS at 37°C (A). NE acts through TLR4. B) Where indicated, macrophages were pretreated with 10  $\mu$ g/ml of control IgG2b or with anti-TLR4 (MTS5) mAb for 30 min in RPMI-FCS and washed before infection. Where indicated (+), purified human NE was added at 200 ng/ml after removal of antibodies and before addition of the parasites. The experiments were performed in triplicate and repeated at least 3 independent times. The graphs show 1 representative experiment. The graphs show means  $\pm$  sd. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001.

production of TGF- $\beta$  (Fig. 4C) and increased NO production, determined as nitrite concentration, (Fig. 4D) in the liver of *ela2*<sup>-/-</sup> mice as compared with those of C57BL/6 background mice, whereas the levels of IL-10 (Fig. 4E) and of IL-6 (unpublished results) were unchanged in *ela2*<sup>-/-</sup>. The cytokine levels were similar in cultures exposed or not to total *Leishmania* Ag, used to assess the parasite-specific response. In the spleen, there was a modest but significant decrease in the levels of IL-10 in *ela2*<sup>-/-</sup> mice as compared with C57BL/6 mice (Fig. 4F), whereas the levels of IL-6 and TGF- $\beta$  were unchanged (unpublished results). These results suggest that lack of NE is associated with detectable changes in the inflammatory and anti-inflammatory responses in organs targeted by *L. donovani* *in vivo*.

Considering that NE seems important for *L. donovani* infection and that we were unable to detect ISP2 in *L. donovani* lysates, we asked if low or absent expression of functional ISP2 was necessary to allow the NE-TLR4 axis to be operational and ensure parasite survival and intracellular growth. To address this, we generated transgenic *L. donovani* expressing the *L. major* ISP2 gene by introducing it into the ribosomal locus of *L. donovani* MW897 (Fig. 5A). Expression of ISP2 in the transgenic line was confirmed by Western blot analysis, in which ISP2 levels were readily detected in 2 independently isolated *L. donovani*:ISP2 clones (Fig. 5B). We confirmed that the expression of ISP2 was maintained in the amastigote stage of the transgenic lines, whereas it was not detected in WT *L. donovani* amastigotes (Fig. 5C). Mice infected with either of the *L. donovani*:ISP2 clones displayed 100-fold lower parasite burdens in comparison with mice infected with WT *L. donovani* (Fig. 5D), indicating that the transgenic parasites are less fit to survive *in vivo*. Next, the ISP2-transgenic *L. donovani* were tested in macrophage infections to address the underlying molecular mechanism. As expected, WT *L. donovani* multiplied inside C57BL/6 macrophages, as assessed by parasite numbers at 72 h (Fig. 6A). In contrast,

*L. donovani*:ISP2 did not increase in numbers by 72 h, suggesting that expression of ISP2, at least at detectable levels, disturbs parasite development in macrophages (Fig. 6A). We tested whether lack of NE activity was responsible for the poor intracellular growth of *L. donovani*:ISP2 by adding exogenous NE to infected C57BL6 macrophages (Fig. 6B). Exogenous NE partially recovered the intracellular development of *L. donovani*:ISP2 at 72 h, suggesting that the negative impact of ISP2 expression is conferred through the reduction in the levels of active NE. Next, we evaluated the involvement of TLRs in *L. donovani*:ISP2 infections. We found that, whereas parasites did not increase in numbers from 3 to 72 h in C57BL6 macrophages, *L. donovani*:ISP2 displayed a more pronounced phenotype in *tlr2*<sup>-/-</sup> macrophages [i.e., the parasite burdens in *tlr2*<sup>-/-</sup> at 72 h were decreased by half in relation to the number internalized in 3 h (Fig. 6C)]. The same trend was observed in macrophages of *tlr4*<sup>-/-</sup> mice, in which at 72 h, there were fewer parasites in comparison to the number of parasites internalized at 3 h (Fig. 6D). These data indicate that *L. donovani* expressing ISP2 display an even more pronounced decrease in fitness in macrophages lacking either TLR2 or TLR4. To further verify an association between the NE-TLR4 axis and *L. donovani* infection in primary macrophages, we performed confocal microscopy (Fig. 7). We found marked colocalization between NE and TLR4 in C57BL/6 macrophages that were in close proximity to intracellular *L. donovani* in both recently internalized promastigotes (Fig. 7A, B) and in amastigotes present at 72 h postinfection (Fig. 7C, D). In parallel, we could also observe parasites that did not colocalize with NE-TLR4. NE-TLR4 colocalization with *L. donovani*:ISP2 was more evident than with WT parasites and was likewise detected at 3 h (Fig. 7E, F) and at 72 h postinfection (Fig. 7G, H). These data are consistent with a role for the TLR4-NE axis in the intracellular development of *L. donovani* in macrophages.

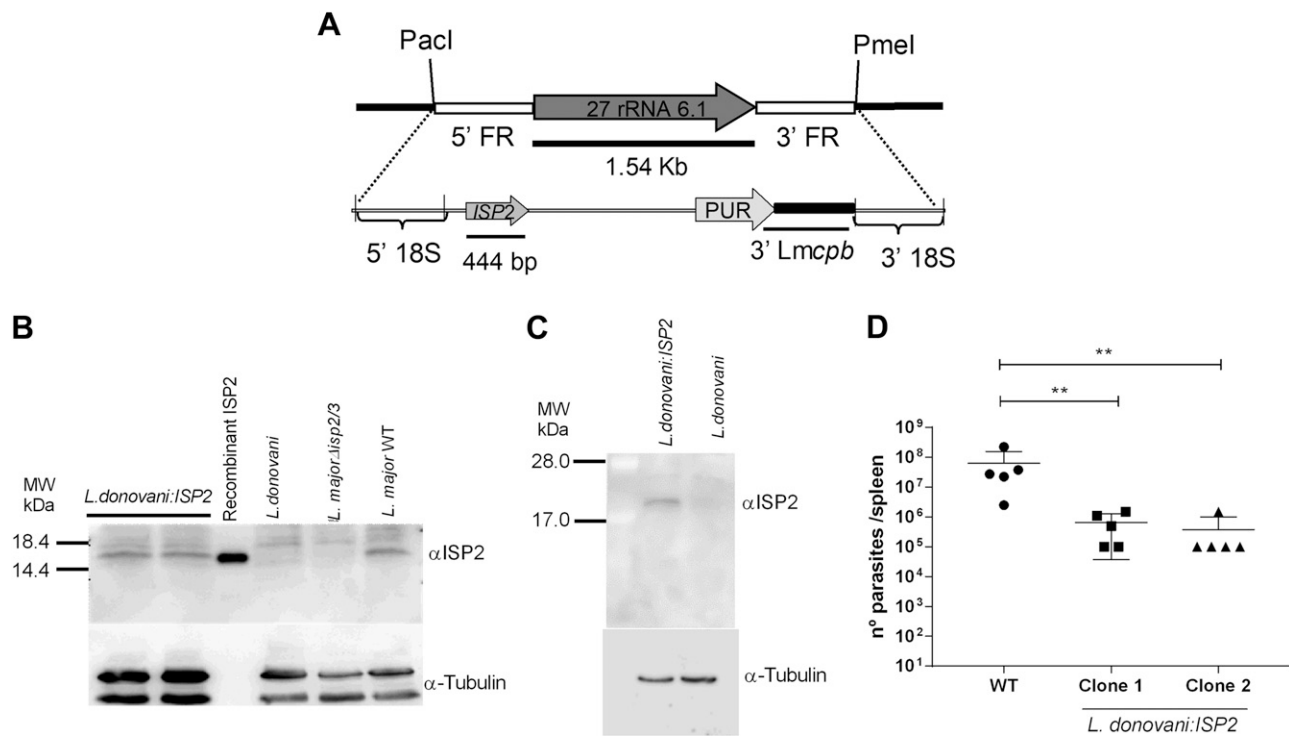
**Figure 4.** NE contributes to *L. donovani* infections *in vivo* and to the anti-inflammatory immune response. *A, B*) Stationary-phase promastigotes were injected in C57BL/6 mice or *ela2*<sup>-/-</sup> mice ( $3 \times 10^7$  parasites/animal,  $n = 6$ /group) in PBS. After 7 d, parasite load in spleens and livers was assessed by limiting dilution. Statistical analyses were performed using 2-way ANOVA. *C–F*) Cellular populations of liver macerates (*C–E*) (ammonium-chloride-potassium solution treated) or splenocytes (*F*) were cultivated in RPMI-FCS at 37°C for 48 h, and the amount of IL-10 and TGF- $\beta$  was determined by ELISA. Where indicated (Ag +), total *L. donovani* Ag (freeze-thawed parasite lysates) were added to the cultures. *D*) The nitrite levels in supernatants of liver macerates were assessed by the Griess method. Experiments were repeated at least 3 independent times, and graphs show 1 representative experiment. The graphs show means  $\pm$  SD. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .



NO and reactive oxygen species (ROS) are important mediators of leishmanicidal activity in macrophages. We investigated if NO and ROS play a role in reduced intracellular survival of *L. donovani:ISP2* at early infection (24 h). The inhibition of NO generation by L-NAME or of superoxide by EUK134 enhanced survival of WT parasites by  $\sim$ 3-fold, suggesting that, as previously described by Phillips *et al.* (30), some degree of early innate killing is associated with the production of nitrogen and ROS (Fig. 8A, B, white bars). However, the reduced survival of *L. donovani:ISP2* at 24 h remained unchanged after treatment with the iNOS inhibitor or the superoxide scavenger,

suggesting that those mediators are unlikely to be the main mechanism of elimination of *L. donovani:ISP2* in the early macrophage infection (Fig. 8A, B, gray bars). Previous work using splenic-derived macrophages (M14.4 line) has attributed killing of *L. donovani* (LV9 strain) in the first 12 h to NO, whereas later killing was attributed to IFN-I (30). We then tested the effect of the IFN-I inducer, poly:IC, in infected C57BL6 macrophages (Fig. 8C, D). Poly:IC provoked a remarkable decrease in the number of intracellular WT *L. donovani* at 72 h (Fig. 8C), whereas it did not further reduce the already low intracellular burdens of *L. donovani:ISP2* (Fig. 8D).





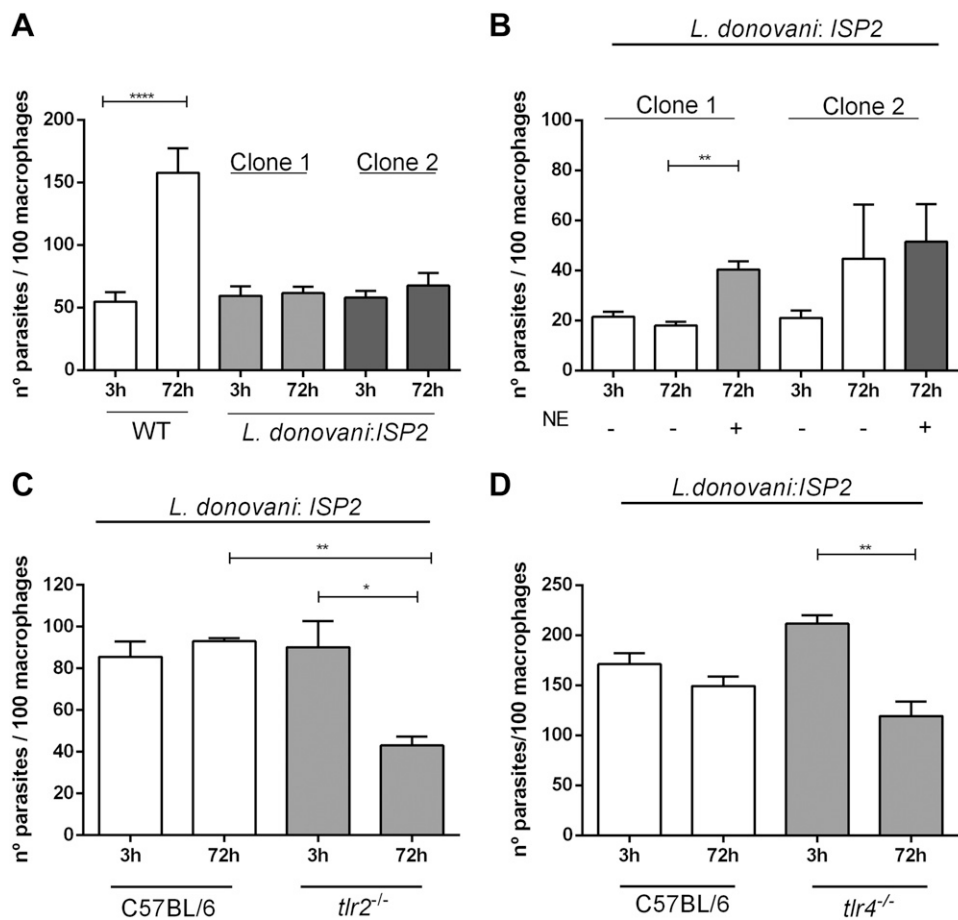
**Figure 5.** Generation of *L. donovani* transgenic lines expressing *L. major* *ISP2*. **A)** Schematic representation of the rRNA locus and the cassette used for insertion of the *ISP2* gene of *L. major* in the genome of *L. donovani*. FR, flanking region; *Lmcpb*, *L. mexicana* cysteine peptidase B; PUR, puromycin resistance gene. Dashed lines indicate the region of gene replacement by homologous recombination (**B**). Two clones of the transgenic parasites (*L. donovani:ISP2*) were checked by Western blot for *ISP2* expression in comparison with parental *L. donovani*, WT *L. major*, and *L. major* line knockout for the *ISP2* and *ISP3* genes ( $\Delta isp2/isp3$ ). Promastigote ( $2 \times 10^7$ ) lysates were probed using antiserum raised to recombinant *ISP2*. Recombinant *L. major* *ISP2* (*ISP2r*) was used as a positive control. **C)** *L. donovani* WT or *L. donovani:ISP2* promastigotes were differentiated into extracellular amastigotes in Schneider's medium, pH 5.4, supplemented with FCS 20% at 37°C for 6 d. In total,  $2 \times 10^7$  amastigotes were washed twice in PBS and lysed in SDS-PAGE sample buffer in reducing conditions, and *ISP2* expression was assessed by Western blot using antiserum raised against recombinant *L. major* *ISP2*. Antibodies to tubulin were used as a loading control. **D)** Stationary-phase promastigotes were injected in C57BL/6 mice ( $3 \times 10^7$  parasites/animal,  $n = 5$ /group) in PBS. After 7 d, parasite load was measured in spleen by limiting dilution. The experiment was repeated 2 independent times, and the graph shows 1 representative experiment. Statistical analyses were performed using 2-way ANOVA. \*\* $P < 0.01$ .

The production of IFN-I (IFN- $\alpha$  or IFN- $\beta$ ) is associated with the activity of the IFN regulatory transcription factors (IRFs) (31). In agreement with that, we showed previously that in macrophage infections with *L. major* *ISP2* gene deletion mutants, there are increased levels of nuclear IRF3 and the production of IFN- $\beta$  by macrophages, leading to parasite killing (19). Likewise, Poly:IC-dependent killing of *L. donovani* LV9 after 12 h was associated with the production of IFN- $\alpha$  (30). Next, we verified the induction of IFN-I expression by qPCR. In C57BL6 macrophages, we did not detect a significant increase in mRNA for IFN- $\alpha$  (Fig. 9A). Surprisingly, infection with WT *L. donovani* induced IFN- $\alpha$  in *ela2*<sup>-/-</sup> macrophages at 2 h, and at 6 h we detected a >10-fold increase in IFN- $\alpha$  mRNA as compared with uninfected cells (Fig. 9B). *L. donovani:ISP2* also induced IFN- $\alpha$ , even though the levels were slightly lower at 6 h in comparison to cells infected with WT parasites. The dynamics of IFN- $\beta$  induction were different: up-regulation of IFN- $\beta$  could only be detected in C57BL6 macrophages at 6 h postinfection (>20-fold increase) with WT parasites, but it was apparent (about 10-fold increase) at 2 h postinfection with *L. donovani:ISP2* and maintained at 6 h (Fig. 9C). Those observations reveal that although *L.*

*donovani:ISP2* can induce IFN- $\alpha$  similarly to WT parasites, it induced about 50% less IFN- $\beta$  in comparison to WT *L. donovani*. The putative association between NE and induction of IFN- $\beta$  was assessed upon infection of *ela2*<sup>-/-</sup> macrophages (Fig. 9D). We found that the induction of IFN- $\beta$  mRNA was partially dependent on NE, once infected *ela2*<sup>-/-</sup> macrophages expressed about 50% less IFN- $\beta$  mRNA either with WT parasites (~12-fold) or *L. donovani:ISP2* (about 5-fold), as compared with C57B6 counterpart macrophages (Fig. 9D).

Because IFN- $\alpha$  was previously associated with the killing of intracellular *L. donovani* LV9 (30) and we observed its induction in 6 h in *ela2*<sup>-/-</sup> macrophages, we tested whether this cytokine influenced intracellular survival (24 h) of *L. donovani* MW897. Addition of exogenous IFN- $\alpha$  at different concentrations did not reduce the survival of WT *L. donovani* in macrophages of either C57BL6 (Fig. 10A) or *ela2*<sup>-/-</sup> (unpublished results). Conversely, IFN- $\beta$  was described as beneficial for the growth of *L. amazonensis* in macrophages (32), whereas it contributes to the killing of *L. major* in macrophages *in vitro* (19). Therefore, we asked whether IFN- $\beta$  was necessary for the intracellular growth of *L. donovani* and whether the low-level induction of IFN- $\beta$  in

**Figure 6.** A) *L. donovani*:ISP2 cannot grow in macrophages, because of lack of NE activity. Thioglycolate-recruited peritoneal macrophages from C57BL/6 mice were cultivated overnight in RPMI supplemented with 10% FCS. The cultures were washed and incubated with stationary-phase promastigotes at a 5:1 parasite:macrophage ratio in RPMI supplemented with 0.1% BSA at 37°C. After 3 h of interaction, the monolayers were washed for the removal of extracellular parasites fixed and stained with Giemsa. For the survival assays, the macrophages were infected as previously described for 3 h, and after the removal of extracellular parasites, the cells were cultured at 37°C in RPMI supplemented with 10% FCS for 72 h. B) Where indicated (+), purified NE was added at 200 ng/ml to macrophages after the 3 h infection and the removal of extracellular parasites and kept in the cultures to 72 h at 37°C in RPMI supplemented with 10% FCS. C, D) Thioglycolate peritoneal macrophages from C57BL/6, TLR4 knockout (*tlr4*<sup>-/-</sup>), or TLR2 knockout mice (*tlr2*<sup>-/-</sup>) were cultivated overnight in RPMI supplemented with 10% FCS. The cultures were washed and incubated with stationary-phase promastigotes at a 5:1 parasite:macrophage ratio in RPMI supplemented with 0.1% BSA at 37°C. After 3 h, the monolayers were washed, fixed and stained with Giemsa, or cultured at 37°C in RPMI supplemented with 10% FCS for 72 h. The experiments were performed in triplicates 2 independent times. The graphs show 1 representative experiment in triplicate. Statistical analyses were performed using 1-way ANOVA and the Bonferroni posttest. \**P* < 0.05, \*\**P* < 0.01, \*\*\*\**P* < 0.0001.



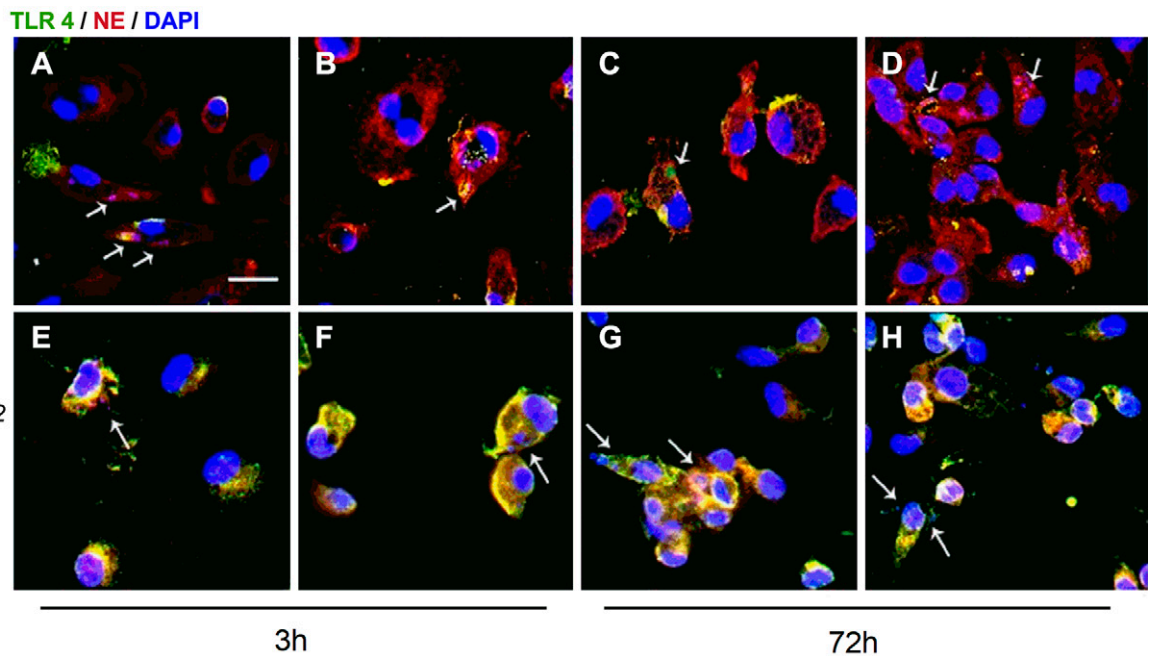
*ela2*<sup>-/-</sup> macrophages could be responsible for the poor parasite development in the absence of NE. This was directly assessed by addition of exogenous IFN- $\beta$  to infected cultures (Fig. 10B). Exogenous IFN- $\beta$  did not affect parasite numbers at 72 h in WT macrophages, whereas it fully restored parasite growth in *ela2*<sup>-/-</sup> macrophages (Fig. 10B). Likewise, addition of exogenous IFN- $\beta$  recovered the intracellular growth of *L. donovani*:ISP2 in both C57B6 macrophages (Fig. 10C) and *ela*<sup>-/-</sup> macrophages (Fig. 10D), linking NE to IFN- $\beta$  production and to parasite survival and growth in macrophages. Moreover, the prevention of the activation of the NE-TLR axis by parasite ISP2 was detrimental for this viscerotropic species.

## DISCUSSION

In humans, *L. donovani* but not *L. major* disseminates and multiplies in visceral organs after dermal inoculation. Experimental mouse models of VL adopt intravenous injection of *L. donovani* or *L. infantum* to bypass the initial barrier and lead to colonization of visceral organs with high parasite burden (33). Here, we used C57BL/6 mice injected with late-stage promastigotes as a model for

visceral infection and detected colonization of liver and spleen up to at least 30 d.

Using NE knockout mice, we showed that NE is required for efficient early liver parasitism and also for the successful development of *L. donovani* MW897 in macrophages *in vitro*. The positive effect of NE on macrophage infection required TLR4 because NE-mediated parasite growth was abolished by anti-TLR4 antibodies. Furthermore, NE was required for the >20-fold increase in the expression of IFN- $\beta$  mRNA in macrophages infected by *L. donovani*, which was necessary for parasite growth, and this was counteracted by the parasite-expressed ISP2. Those observations suggest that ISP2 expression might pose a disadvantage to viscerotropic *Leishmania* species because it prevents the activation of the NE-TLR platform, at least in macrophages. In fact, the *ISP2* gene is disrupted in the genome of the *L. donovani* reference strain BPK282A1 (LDBPK\_150530.1), and we could not detect expression of the ISP2 protein in *L. donovani* MW897 used in this study, even though the gene is intact. *Leishmania* species have 3 single copy genes showing homology to ecotins: *ISP1*, *ISP2*, and *ISP3*, and *L. major* expresses *ISP1* and *ISP2* proteins in promastigotes, whereas *ISP3* is not expressed (18, 34). Previously, we showed that *L. major* *ISP1* does not inhibit



**Figure 7.** Colocalization of NE and TLR4 with *L. donovani* in infected macrophages. Macrophages of C57BL/6 mice were infected with WT *L. donovani* or *L. donovani*:ISP2 (clone 2) for 3 h in RPMI-BSA and processed for immunofluorescence or washed and cultured for 72 h in RPMI-FCS and processed for immunofluorescence. Coverslips were treated with anti-TLR4 antibodies washed and incubated with anti-rabbit–Alexa 488. Coverslips were subsequently incubated with antibodies to NE and washed and incubated with anti-rabbit–cyanine 3 antibodies. Coverslips were treated with DAPI for 5 min and washed and mounted for immunofluorescence. Samples were analyzed by confocal microscopy. White arrows point to the parasite. Scale bar, 2  $\mu$ m.

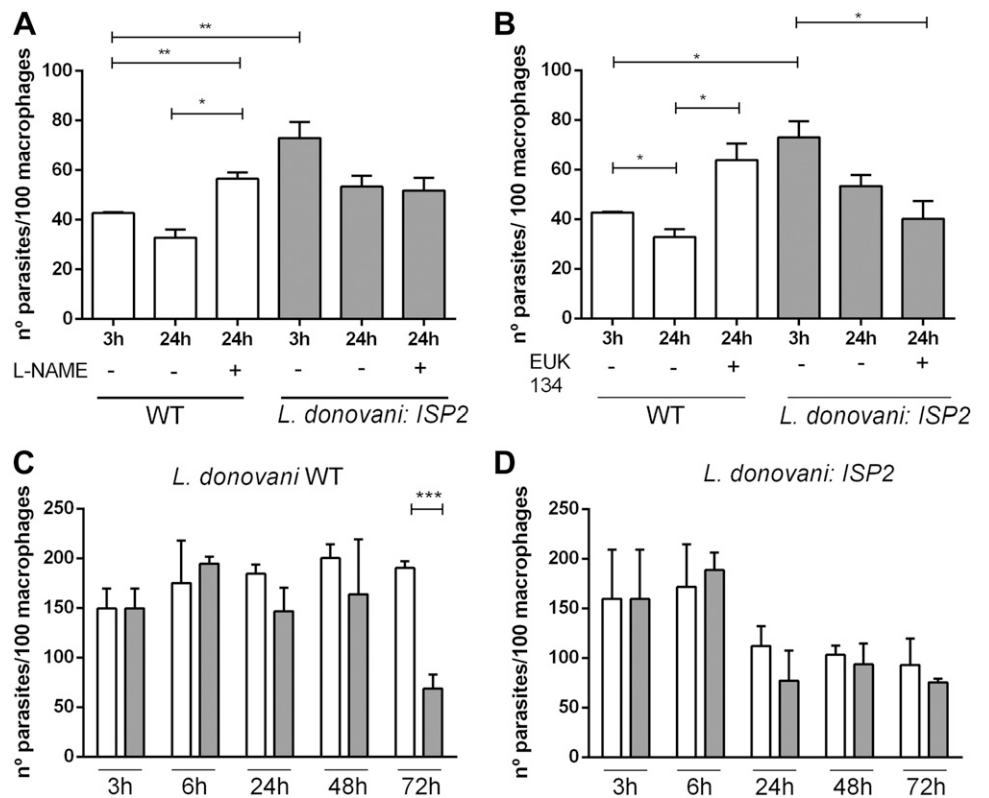
trypsin, is 10-fold less potent than ISP2 for the inhibition of NE, and does not influence macrophage infection; thus, ISP1 is unlikely to prevent NE-TLR activation (34). Low levels or lack of ISP2 expression could reflect an adaptation of *L. donovani* MW897, allowing the NE-TLR signaling axis to remain functional in macrophages. In contrast, evidence for the expression of ISPs in another *L. donovani* strain was provided in a study in which an  $\sim$ 21.8 kDa protein that inhibits trypsin and NE was purified from an Indian strain of *L. donovani* (24). Notably, even though this strain was isolated from an infected individual, the authors stated that it became avirulent after several *in vitro* passages. Mass spectrometry analysis of the purified protein matched ISP2 from *L. infantum*. Immunostaining revealed LdISP close to the flagellar pocket of *L. donovani* strain AG83 promastigotes, which is compatible with putative secretion of ISP for the inhibition of the NE at the surface of macrophages (25). In another study, recombinant LdISP2 was reported to inhibit the complement-related serine peptidase mannan-binding lectin serine protease (MASP)-2, whereas it did not inhibit MASP-1 or the C1 complex (26). However, inhibitory activity was only observed at nonphysiologic concentrations of recombinant LdISP2 ( $>5 \mu$ M), and inhibition of C4b formation was only achieved at  $>10 \mu$ M of LdISP2, suggesting that those peptidases are unlikely to be a target of ISP2 *in vivo*.

The requirement of an active NE-TLR pathway in infections by *L. donovani* MW897 supports the hypothesis that ISP2 reduces parasite fitness in macrophages. Indeed, when we expressed the *L. major* ISP2 in *L. donovani* MW897, the transgenic line failed to grow inside macrophages. Growth was restored by addition of exogenous NE, therefore linking inhibition of NE by ISP2 to the

impairment of *L. donovani* development in macrophages. Further, the complementation of the intracellular growth phenotype by exogenous NE revealed that the transgenic line does not have an intrinsic growth defect. These data reveal a striking difference between *L. donovani* and *L. major* in the molecular mechanism by which the different species survive and grow inside macrophages.

Previous experimental infections of TLR knockout mice with *L. donovani* suggested that TLR2 and TLR4 play antagonizing roles in which TLR4 is essential to control parasite burden (9), but the underlying mechanisms and target cells mediating those roles were not explored. TLR4 signaling was also shown to influence immunity by down-regulating IL-10 and controlling parasite burden (35). Even though we found that macrophage infection *in vitro* by *L. donovani* requires TLR4 for parasite growth, it is likely that TLR4 present in additional cell types besides macrophages plays a role during experimental infection, contributing to parasite control. Using a monocytic cell line RAW or bone marrow–derived macrophages, Srivastav *et al.* (36) reported that *L. donovani* AG83 deactivates the TNF receptor-associated factor (TRAF)6–interleukin-1 receptor-associated kinase (IRAK)1 (TRAF6-IRAK1) complex during long-term infection and sustains elevated levels of the TLR-negative regulator IRAK-M. Even though *L. donovani* was found to enhance the stability of the IRAK1-myeloid differentiation primary response 88 (MyD88) complex, thus potentially deactivating TLR-mediated responses, the consequences of the direct activation of TLR2 or TLR4 in infected macrophages were not directly addressed. Using macrophages derived from bone marrow of BALB/C, Gupta *et al.* (37) reported

**Figure 8.** ROS or NO are not responsible for killing of *L. donovani:ISP2*. Macrophages of C57BL/6 mice were infected with WT *L. donovani* or *L. donovani:ISP2* for 3 h in RPMI-BSA and washed and further cultured in RPMI-FCS for 24 h before fixation and staining. **A, B**) Where indicated (+), immediately after the 3 h infection and washing, the cultures were treated with 1 mM of the inhibitor of iNOS, L-NAME (A), or 50  $\mu$ M of the scavenger of ROS, EUK134 (B). **C–E**) Modulation of type I IFN responses affect *L. donovani* growth in macrophages. Thioglycolate-recruited macrophages from C57BL/6 mice were cultured overnight, washed, infected with *L. donovani* WT (C) or *L. donovani:ISP2* (D) for 3 h in RPMI-BSA, washed, and fixed (3 h) or further cultured (24–72 h) in RPMI-FCS (white bars). Alternatively, after the 3 h infection and washing, cultures were treated with poly(I:C) (25  $\mu$ g/ml) (gray bars) and further cultured in RPMI-FCS for up to 72 h. Experiments were performed in triplicate at least 2 independent times. The graph shows 1 representative experiment. Statistical analyses were performed using 1-way ANOVA and the Bonferroni posttest (A, B) or multiple Student's *t* tests with the false discovery rate posttest (C, D). \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

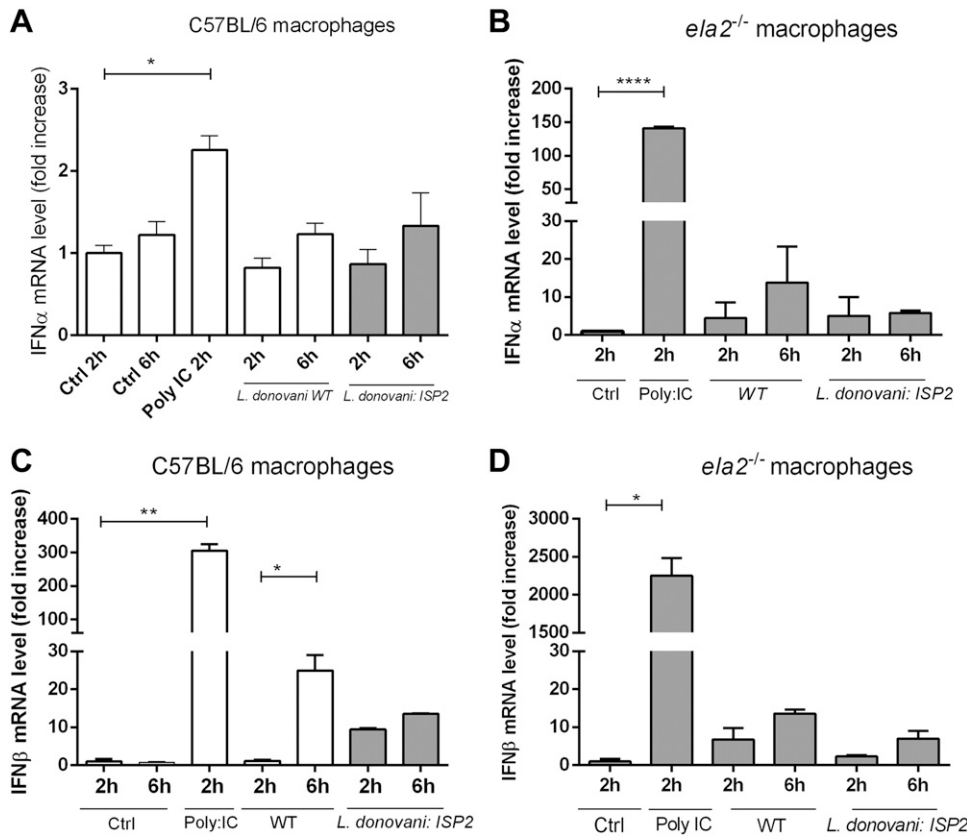


suppression of TLR4 when the cells were concomitantly challenged with LPS and *L. donovani* AG83. Both studies propose that suppression of TLR4 is required for successful macrophage infection by *L. donovani* AG83. However, the latter used LPS stimulation as a means to address TLR4 cellular deactivation by *L. donovani* but not the direct participation of TLR4 in parasite infection in the absence of additional external stimuli. Although several studies conclude that the engagement of TLR4 is leishmanicidal, our results clearly show that, at least in *in vitro* macrophage infections, both TLR2 and 4 are essential for parasite intracellular replication. It is possible that the downstream signals conveyed by the NE-TLR4 platform during parasite uptake, in combination with TLR2 mediated responses, lead to downstream effectors which differ, at least in part, from LPS-induced responses. Thus, it is feasible that the parasite has adapted to prevent activation of TLR4-MyD88-IRAK-mediated responses, whereas TLR4-TIR-domain-containing adapter-inducing interferon  $\beta$  (TRIF) responses remain intact.

Even though we observed colocalization between NE and TLR4, we do not know how the NE-TLR4 signals are orchestrated at the macrophage surface during parasite uptake. Stimulation of TLR4 by NE is described in other cell types, leading to IRAK-TRAF6 responses (15). In such a model, stimulation of TLR4 is achieved indirectly through NE-dependent activation of additional surface peptidases and cleavage of epidermal growth factor, leading to TLR interaction with epidermal growth factor receptors (38). It is possible that NE also acts on TLRs indirectly during

parasite uptake. We observed colocalization of NE and TLR4 in the parasitophorous vacuole, even at 72 h post-infection, suggesting that the parasite recruits the NE-TLR4 (and perhaps NE-TLR4-TLR2) complex to the phagosome, and it may remain active in the parasitophorous vacuole during amastigote intracellular growth. In agreement with a protective role for TLR2 in the phagosome, it was recently described that TLR2 is required to delay phagosome maturation after *L. donovani* uptake, contributing to parasite survival (10). It is likely that *L. donovani* MW897 mobilizes the NE-TLR4-TLR2 pathways during phagocytosis, leading to alternative downstream events and committing the host cell to unresponsiveness to otherwise classic TLR stimuli. Of note, in RAW cells costimulated with LPS and *L. donovani*, TRAF3 ubiquitination and degradation is disrupted by the parasite, down-regulating LPS-related TLR4 downstream responses such as TNF- $\alpha$  and IL-12 (37). Interestingly, the authors noticed that TRAF3 ubiquitination linked to K63, which is related to IL-10 induction through MyD88-independent and TRIF-dependent pathway, is preserved in *L. donovani*-infected macrophages. This is in agreement with our observation that the NE-TLR4 axis promotes IFN- $\beta$  production, which is likewise defined as dependent on the TRIF pathway (39).

Previous works identified IFN- $\beta$  as a key factor for the growth of *L. amazonensis* and *L. braziliensis* in macrophages because it helps to control superoxide production *via* an increase in superoxide dismutase 1 (32, 40). Here, when we added exogenous IFN- $\beta$ , we restored the growth of *L. donovani:ISP2* in macrophages and that of WT *L. donovani* in



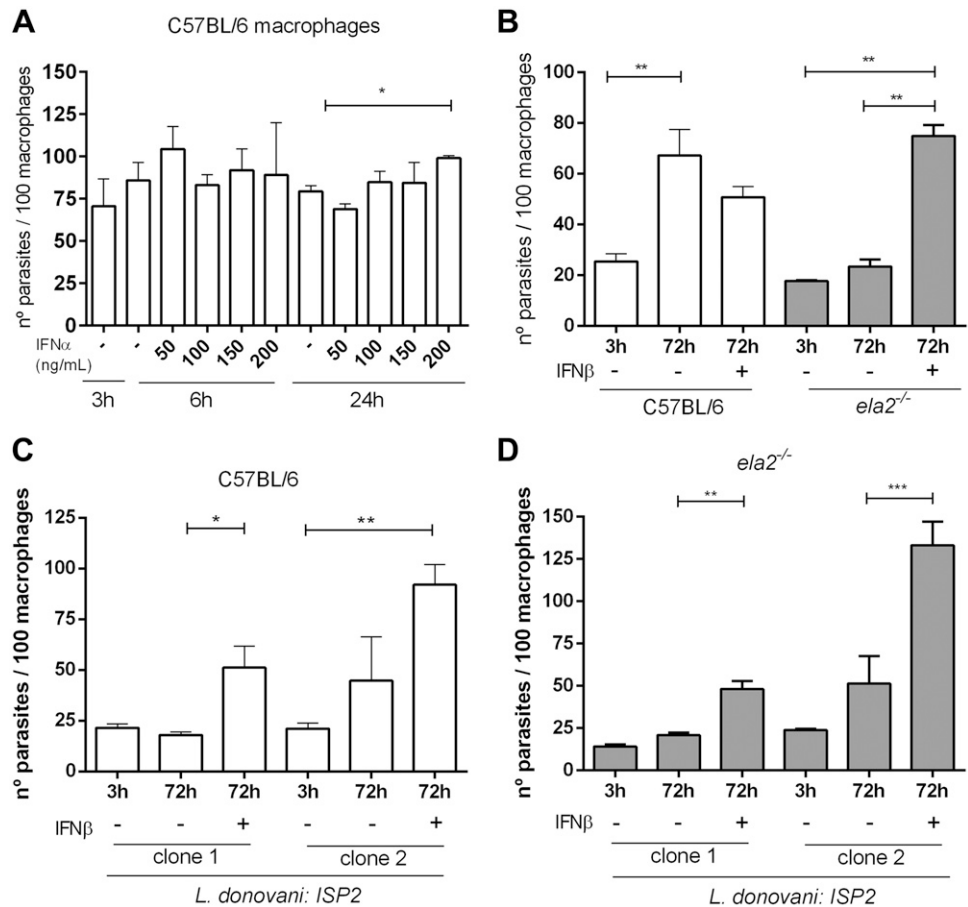
**Figure 9.** NE contributes to the induction of IFN- $\beta$  in macrophages infected with *L. donovani*. Thioglycolate-recruited macrophages from C57BL/6 mice (A, C) or *ela2*<sup>-/-</sup> mice (B, D) were cultured overnight, washed, and infected with WT *L. donovani* or *L. donovani*:ISP2 for 2 or 6 h, and cultures were washed, total RNA was extracted, and the cDNA samples were used as templates in qPCR for the determination of the relative mRNA levels for IFN- $\alpha$  (A, B) and IFN- $\beta$  (C, D). Uninfected cells were used as control levels, and cells pretreated with 25  $\mu$ g poly:IC were used as positive control. Ctrl, control. The experiments were performed 3 independent times in 2 biologic replicates each time. The graphs show 1 representative experiment in duplicate. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

*ela2*<sup>-/-</sup> macrophages, showing that the requirement for NE for parasite growth can be bypassed by IFN- $\beta$ . We observed that NE is a crucial element to induce the high-level expression of IFN- $\beta$  in macrophages infected with *L. donovani* MW897 in the absence of additional stimuli, as *ela2*<sup>-/-</sup> macrophages displayed 50% less induction of IFN- $\beta$  when compared with WT macrophages. However, NE-deficient macrophages were still capable of inducing IFN- $\beta$  to the same levels as WT macrophages when exposed to poly:IC, a classic activator of IFN-I, supporting the concept that NE-requirement for type I IFN induction is specific to the parasite:macrophage interaction. This aligns well with our observation that *L. donovani* but not *L. donovani*:ISP2 provoked increase in the nuclear levels of IRF3 in macrophages (unpublished results), linking NE activity to the expression of type I IFN genes. This was further supported by qPCR data showing that IFN- $\beta$  mRNA could not be induced in NE knockout macrophages to the same levels as observed in WT macrophages. However, we still detected a significant level of IFN- $\beta$  induction (about a 10-fold increase), revealing that additional stimuli other than NE-TLR4 contribute to IFN- $\beta$  induction, such as the previously described lipophosphoglycan induction *via* TLR2 (32). It is tempting to speculate that viscerotropic strains have adapted to require very high levels of IFN- $\beta$ , which can be achieved through 2 simultaneous signals conveyed *via* TLR2 and NE-TLR4. This aligns well with our observation that the supplementation of IFN- $\beta$  to the infected cultures restored growth-deficient phenotypes observed throughout this study. It would be interesting to investigate whether the NE-induced IFN- $\beta$  requirement for

intracellular growth is a common trait for other *L. donovani* strains, including the reference strain in which the ISP2 gene is truncated. Recently, a role for IFN- $\beta$  in promoting metastatic spread of *L. guyanensis* and relapse of infection was reported, associating antiviral responses to disease sustenance and severity (41). It is tempting to speculate that production of IFN- $\beta$  is a common trait between *Leishmania* sp. required for parasite dissemination.

Interestingly, we found virtually no induction of the other IFN-I, IFN- $\alpha$ , in C57BL6-infected macrophages but significant induction in *ela2*<sup>-/-</sup> macrophages. IFN- $\alpha$  and IRF7 were implicated in the innate killing of *L. donovani* LV9: parasites were rapidly killed in RAW or 14M1.4 macrophage cell lines in a 2-stage process relying on early production of NO ( $\leq 6$  h) and later production of IFN- $\alpha$ , dependent on IRF7 (30). More specifically, addition of exogenous IFN- $\alpha$  accelerated the killing of intracellular *L. donovani* LV9 in infected macrophages (30). In our model, exogenous IFN- $\alpha$  did not influence parasite survival or growth. The discrepancy between our data and those reported by Phillips *et al.* (30) may be due to the different origins of macrophage populations and *L. donovani* strains used in the studies. In liver macrophages (Kupfer cells), IRF7 does not affect amastigote uptake or early survival, whereas it was necessary to mount inflammatory T-cell-mediated immunity and NO production by infected macrophages (42). Our results indicate that NO and ROS played a small role in the innate killing of *L. donovani* in inflammatory peritoneal macrophages and are not implicated in the early death of ISP2-expressing *L. donovani*. Even though the addition of poly:IC classically leads to type I IFN production

**Figure 10.** IFN- $\beta$  restores the intracellular growth of *L. donovani*. Macrophages of C57BL/6 or of *ela2*<sup>-/-</sup> mice were infected with *L. donovani* (A, B) or *L. donovani:ISP2* (C, D) for 3 h and washed; recombinant IFN- $\alpha$  (A) or recombinant IFN- $\beta$  (1000 U/ml) (B–D) was added to the cultures; and the cells were maintained in RPMI-FCS at 37°C for the indicated times. The cultures were fixed and Giemsa stained. The number of intracellular parasites was estimated by counting under the light microscope. The experiments were performed 3 independent times, in triplicate. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.



and should, in theory, help to promote parasite growth through induction of IFN- $\beta$ , this stimulus has pleiotropic effects on macrophages, and additional factors might have overwhelmed the potential protective effect of IFN- $\beta$ .

In summary, we showed that *L. donovani* hijacks the NE-TLR4 pathway of macrophages in order to contribute to IFN- $\beta$  production at levels that ensure parasite survival and intracellular growth. How NE and IFN- $\beta$  influence the course of the infection and their potential impact on visceral pathology remain to be elucidated. [FJ]

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## AUTHOR CONTRIBUTIONS

J. C. Mottram and A. P. C. A. Lima conceived the project and directed experimental work and data analyses; B. T. Dias performed the majority of the experimental work; K. L. Dias-Teixeira and T. Calegari-Silva performed qPCR experiments and data analyses; J. P. Godinho performed immunofluorescence experiments; M. S. Faria provided key guidance assistance and experimental assistance; U. Lopes contributed to data analyses and critical discussions; M. M. Mukhtar provided the *L. donovani* isolate; and M. M. Mukhtar, U. Lopes, J. C. Mottram, and A. P. C. A. Lima wrote the manuscript.

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