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PLGA nanoparticles loaded with KMP-11 stimulate innate immunity and induce the killing of *Leishmania*

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Abstract

We recently demonstrated that immunization with polyester poly(lactide-co-glycolide acid) (PLGA) nanoparticles loaded with the 11-kDa *Leishmania* vaccine candidate kinetoplastid membrane protein 11 (KMP-11) significantly reduced parasite load in vivo. Presently, we explored the ability of the recombinant PLGA nanoparticles to stimulate innate responses in macrophages and the outcome of infection with *Leishmania braziliensis* in vitro. Incubation of macrophages with KMP-11-loaded PLGA nanoparticles significantly decreased parasite load. In parallel, we observed the augmented production of nitric oxide, superoxide, TNF-α and IL-6. An increased release of CCL2/MCP-1 and CXCL1/KC was also observed, resulting in macrophage and neutrophil recruitment in vitro. Lastly, the incubation of macrophages with KMP-11-loaded PLGA nanoparticles triggered the activation of caspase-1 and the secretion of IL-1β and IL-18, suggesting inflammasome participation. Inhibition of caspase-1 significantly increased the parasite load. We conclude that KMP-11-loaded PLGA nanoparticles promote the killing of intracellular *Leishmania* parasites through the induction of potent innate responses.

From the Clinical Editor: In this novel study, KMP-11-loaded PLGA nanoparticles are demonstrated to promote the killing of intracellular *Leishmania* parasites through enhanced innate immune responses by multiple mechanisms. Future clinical applications would have a major effect on our efforts to address parasitic infections.

Key words: *Leishmania*; PLGA; Nanoparticle; Macrophage; Innate response

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The use of particles as a tool for vaccine delivery was based on the favored uptake of particulate, rather than soluble, forms of antigen (review in Storni et al.[1]) and the ability of antigen-loaded particles to act as depots, leading to the slow release of antigen and thereby prolonging antigen availability to the immune system (review in Panyam and Labhasetwar[2]). Antigen-loaded particles are also efficiently taken up by antigen presenting cells (APCs) and are subsequently delivered to secondary lymphoid organs[3] where priming of the adaptive immune response occurs. Among the polymers used for in the production of particles, able to induce protective immune responses is the biodegradable and biocompatible polyester poly(lactide-co-glycolide acid) (PLGA), which has been used in humans as reabsorbable suture material and in delivery systems for the controlled release of drugs.[4]

Immunization with antigen-loaded PLGA particles induces protective immune responses in different experimental models.[5–7] In the field of leishmaniasis, a disease caused by *Leishmania* parasites that infect and multiply within macrophages, immunization with cysteine proteinase entrapped in solid lipid nanoparticles (SLNs)[8,9] or with autoclaved *Leishmania* antigen encapsulated in PLGA nanoparticles[10] conferred protection against disease. Recently, we evaluated the capacity of nanoparticles loaded with the 11-kDa kinetoplastid membrane protein (KMP-11)[11] to confer protection against cutaneous leishmaniasis caused by *Leishmania braziliensis*.[12] A promising vaccine candidate, KMP-11 is highly conserved among trypanosomatids,[13] and KMP-11 stimulation of cells obtained from cured leishmaniasis patients induces IFN-γ production.[14] Vaccination with KMP-11 also prevented disease development in different experimental models of leishmaniasis.[15–17] We demonstrated that mice inoculated with PLGA nanoparticles loaded with plasmid DNA coding for KMP-11 or loaded with KMP-11 recombinant protein developed a cellular immune response. Following challenge with live *L. braziliensis* parasites, immunized mice displayed a significant reduction in the parasite load, an outcome associated with increased levels of IFN-γ and TNF-α.[12]

Given this capacity of KMP-11-loaded PLGA nanoparticles to induce an effective adaptive immune response in vivo, we hypothesized that recombinant nanoparticles also stimulate innate responses in macrophages, the host cell in which *Leishmania* replication occurs. This paper describes the effects of cell stimulation with KMP-11-loaded nanoparticles on infection with *L. braziliensis* parasites—distinguished from other etiological agents of leishmaniasis by its chronicity, latency, and tendency to metastasize in the human host[18] and the mechanisms that are associated with parasite killing.

Methods

Preparation and characterization of KMP-11-loaded nanoparticles

Nanoparticles (NPs) were prepared employing a solvent evaporation process using Total Recirculation One Machine System (TROMS®).[19] Initially, several formulation parameters were assayed in order to achieve high antigen (recombinant KMP-11) loading and monodisperse particles (< 500 nm). The effect of the different formulations and particle characteristics were studied by factorial design. Factors and levels studied were PLGA polymer type [Resomer® 503H co-polymer (PL/GA ratio 50:50, MW 34 kDa carrying uncapped hydroxyl and carboxyl) or Resomer® 756, PL/GA 75:25, MW 98 kDa, Boehringer Ingelheim], which differs in molecular weight, organic solvent (Dicloromethane, DCM, or ethyl acetate, EA, Panreac, Spain), stabilizer (Pluronic F68® alone or Pluronic F68+ cationic lipid DOTAP [(1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (chloride salt) (Avanti Polar Lipids, Inc.)] and antigen amount (2 or 4 mg of rKMP11). The study of formulation factors was conducted with rKMP-11 since previous studies indicated that DNA encapsulation in PLGA particles containing the cationic lipid DOTAP in the organic phase was close to 100%.[20] Different formulations of the nanoparticles loaded with rKMP-11 were developed, enabling optimization of nanoparticles with desired particle size, polydispersity and encapsulation efficiency. Size, size distribution and encapsulation efficiency were evaluated as the responses (Supplemental Material, Table 1). Nanoparticle size and polydispersion were analyzed by photon correlation spectroscopy (Malvern Instruments, UK). The amount of rKMP-11 (rProtein) loaded onto nanoparticles was determined by the Micro BCA Protein Assay (Pierce).

Statistical analysis revealed that among parameters studied, the type of PLGA polymer significantly affected particle size and choice of stabilizer significantly affected the encapsulation efficiency. Other factors (solvent and amount of antigen) did not have any significant effect on the parameters evaluated (Supplemental Material, Fig. 1). Based on these results, nanoparticle formulation was conducted as described.[12] Briefly, 100 mg of lactic and glycolic acid (PLGA) Resomer 503 co-polymer (4% wt/vol) and 10 mg of DOTAP were dissolved in dicloromethane (DCM) and injected onto a Pluronic F68® solution (500 μL 6% wt/vol) containing 2 mg of recombinant KMP-11 (rProtein), wild-type plasmid DNA (pcDNA3) (WT DNA) or pcDNA3 coding for KMP-11 (rDNA). This W_1/O emulsion circulated through the system to homogenize the emulsion droplet size. The preformed emulsion was injected into the outer water (W_2) phase, 15 mL of polyvinylalcohol (PVA, 87% hydrolized, molecular weight 115 000, BDH, UK) (0.5% wt/vol), under a constant pump flow. The resulting double emulsion ($W_1/O/W_2$) was homogenized by circulation through the system. After solvent evaporation under magnetic stirring, the nanoparticles were purified by centrifugation, lyophilized and stored at −20°C. The amount of plasmid DNA (rDNA or WT) loaded onto nanoparticles was determined by a fluorimetric assay (PicoGreen® dsDNA Quantitation Kit, Molecular Probes).[12] To estimate integrity, nanoparticles loaded with plasmid DNA (rDNA or WT DNA) were dissolved with DMSO, and DNA was ethanol precipitated at −80°C. The samples were analyzed by agarose gel electrophoresis. Nanoparticles loaded with KMP-11 (rProtein) were dissolved in methylene chloride. The organic solvent was evaporated, and the residue was resuspended in electrophoresis sample buffer. The samples were subjected to SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250.

In vitro release studies

Nanoparticles (5 mg) loaded with rProtein or with rDNA were dispersed in 1 mL of PBS and maintained under agitation at 37°C. At predetermined intervals, the samples were centrifuged at 27.100 × *g* for 20 min, and the amount of rDNA or rProtein released from the particles into the supernatants was determined. The release data were expressed as the cumulative percentage of rProtein or rDNA of the initial content of the particles versus time.

Mice

Female BALB/c mice (6-8 weeks of age) were obtained from the CPqGM/FIOCRUZ Animal Facility. All mice were maintained under pathogen-free conditions. All animal procedures were approved by the local Ethics Committee on Animal Care and Utilization (CEUA—CPqGM/FIOCRUZ- L-065-8).

Parasite culture

L. braziliensis promastigotes (strain MHOM/BR/01/BA788)[21] were grown in Schneider medium (Sigma-Aldrich) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated fetal calf serum (all from Invitrogen). Stationary-phase promastigotes were used in all experiments.

Infection of PLGA-stimulated macrophages with L. braziliensis

BALB/c mice were injected i.p. with 3% thioglycolate. Five days after injection, peritoneal lavage was performed using 8 mL RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Invitrogen). To obtain monolayers, cells (6×10^5cells/mL) were place into glass coverslips within the wells of a 24-well plate and were left to adhere for 2 h, at 37°C and 5% CO_2. Nonadherent cells were removed by gentle and extensive washing with PBS; purity was routinely above 99% (data not shown). Remaining cells (3×10^5cells/mL) were stimulated with plasmid DNA-loaded nanoparticles (carrying the equivalent of 10 μg/mL of encapsulated rDNA or 10 μg/mL of encapsulated WT DNA), rProtein-loaded nanoparticles (carrying the equivalent of 3 μg/mL of encapsulated recombinant KMP-11) or with unloaded (empty) nanoparticles (847 μg/mL of polymer) in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Invitrogen). After 4 h of stimulation, the cells received 3×10^6 cells/mL of stationary-phase *L. braziliensis* promastigotes and were incubated at 37°C in complete RPMI medium. After 24 h of infection, the glass coverslips containing the infected macrophages were washed and stained with H&E, and the intracellular amastigotes were counted by light microscopy. The results are shown as the percentage of infected cells per 100 macrophages. The number of intracellular amastigotes was counted in 100 macrophages. Alternatively, the infected macrophages were washed extensively, and the medium was replaced with 0.5 mL of Schneider medium (Sigma) supplemented with 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were cultured at 26°C for an additional 5 days, and the number of viable promastigotes was determined with a hemocytometer. In some experiments, the macrophages were pretreated for 24 h with Z-WEHD-FMK (R&D systems) (100 μM) to block caspase-1 activation and were later stimulated with the recombinant nanoparticles as above. Subsequently, the cells were infected with *L. braziliensis* as described and assayed for the percentage of infected macrophages and for the number of intracellular amastigotes.

Table 1
Characterization of nanoparticles formulated with rProtein, WT DNA or rDNA.

Nanoparticle formulation	Size (nm)	Zeta potential (mV)	Molecule loading (μg/mg NP)	% EE[a]
Empty	315 ± 35	30 ± 10	–	–
rKMP11 (rProtein)	370 ± 32	23 ± 7	3.5 ± 0.5	43.7 ± 6.3
pcDNA3 (WT DNA)	373 ± 15	23 ± 9	7.2 ± 0.7	90.3 ± 8.2
pcDNA3-KMP11 DNA (rDNA)	443 ± 75	28 ± 6	6.4 ± 1.2	79.5 ± 14.5

Nanoparticles were produced by the solvent evaporation method. Results represent the mean ± SD of two independent preparations.
[a] EE, encapsulation efficiency.

Production of reactive species

Macrophages (3×10^6 cells/mL) were obtained and stimulated with plasmid DNA-loaded nanoparticles (carrying the equivalent of 10 μg/mL of encapsulated rDNA or 10 μg/mL of encapsulated WT DNA), rProtein-loaded nanoparticles (carrying the equivalent of 3 μg/mL of encapsulated recombinant KMP-11) or with unloaded (empty) nanoparticles (847 μg/mL of polymer) in RPMI medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen), in the presence of LPS (5 ng/mL) for 24 h. The Griess method was used to measure nitric oxide (NO) production via its stable reaction product, nitrite (NO_2^-).[22] For the detection of intracellular reactive oxygen species (ROS), macrophages were also stimulated as described above. Following incubation, 10 μM of the oxidative fluorescent dye probe DHE (dihydroethidium) (Invitrogen) was added to the cultures and cells were further incubated for 30 min at 37°C. The data were acquired and analyzed using a FACSort flow cytometer (BD Immunocytometry) and FlowJo software (Tree Star).

Cytokine and chemokine production

Macrophages (3×10^6 cells/mL) were obtained and stimulated as above for 4 h. The supernatants were collected, and the production of TNF-α, IL-6, IL-10 and CCL2/MCP-1 was evaluated using an inflammatory Cytometric Bead Array (BD Biosciences) following the manufacturer's instructions. The data were acquired and analyzed using a FACSort flow cytometer (BD Immunocytometry) and FCAP Array™ CBA Analysis Software (BD Biosciences). CXCL1/KC was detected by sandwich ELISA (R&D Systems) according to the manufacturer's instructions. For the detection of caspase-1 activity, as

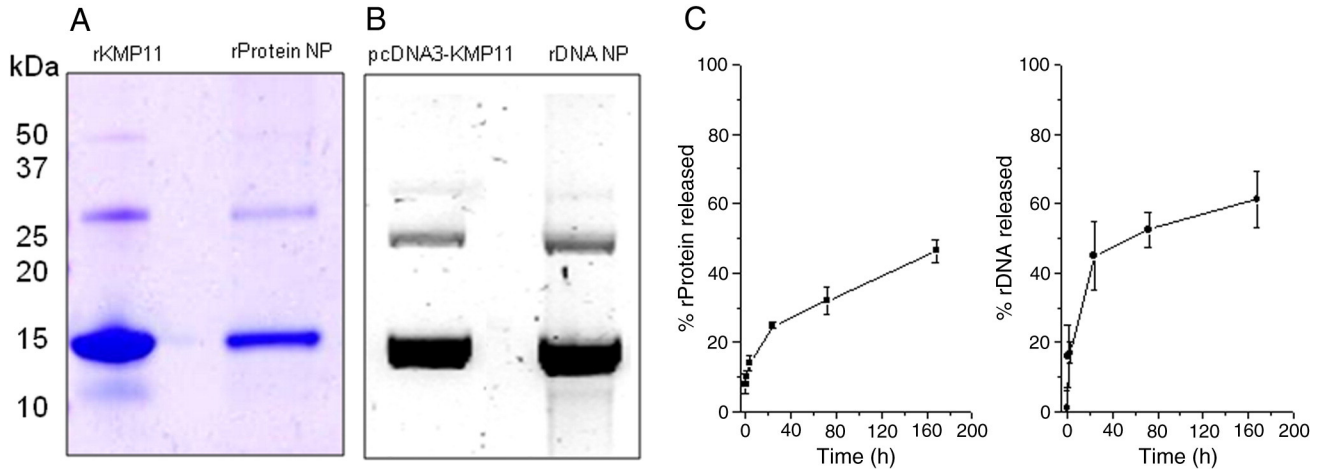


Figure 1. Study of rProtein and rDNA integrity after loading in NP. **(A)** SDS-PAGE of KMP-11 and rProtein nanoparticles extracted with 0.1N NaOH. **(B)** Agarose gel of rDNA alone and rDNA extracted from recombinant nanoparticles (rDNA NPs) and stained with ethidium bromide. **(C)** In vitro release of rProtein and rDNA from nanoparticles over time.

well as the presence of IL-1β and IL-18, experiments were performed in the presence of LPS (5 ng/mL). After 24 h, the caspase-1 activity was measured by a colorimetric assay (R&D Systems) according to the manufacturer's instructions. The supernatants were collected and analyzed by ELISA for the presence of mature IL-1β (E-Bioscience) and IL-18 (Medical & Biological Laboratories). For inhibitory assays, the macrophages were pretreated for 24 h with Z-WEHD-FMK (R&D systems) (100 μM) to block caspase activation. The supernatants were collected and assayed for the presence of mature IL-1β as above. Alternatively, the supernatants were collected, frozen at −20°C and employed in macrophage and neutrophil chemotaxis assays.

Chemotaxis assay

Macrophages were obtained as described above, while neutrophils were obtained following stimulation with 3% thioglycolate for 7 h. Briefly, peritoneal exudate neutrophils obtained 7 h after 3% thioglycolate solution injection were incubated at 37°C/5% CO_2 for 1 h in 250-mL flasks (Costar). Nonadherent cells were stained with anti-Ly-6G to assess purity. Data were acquired and analyzed using a FACSort flow cytometer (BD Immunocytometry) and FlowJo software (Tree Star). According to this protocol, purity of Ly-6G$^+$ cells was routinely above 93% (data not shown). The cells (polymorphonuclear cells or macrophages) were resuspended in complete RPMI 1640 medium (10^5 cells/well) and added to the upper compartment of a Chemo TX System (Neuro Probe). The supernatants of macrophages stimulated with rDNA- or rProtein-loaded nanoparticles or with unloaded (empty) nanoparticles, as described earlier, were added to the bottom compartment. The plates were incubated for 90 min (macrophages)[23] or 60 min (polymorphonuclear cells)[24] and migration in the presence of culture medium (random chemotaxis) was used as a negative control. Chemotaxis indices were calculated as the ratio of the number of cells that migrated in the presence of the stimulus to the number of cells that migrated following stimulation with culture medium alone.[25]

Statistical analysis

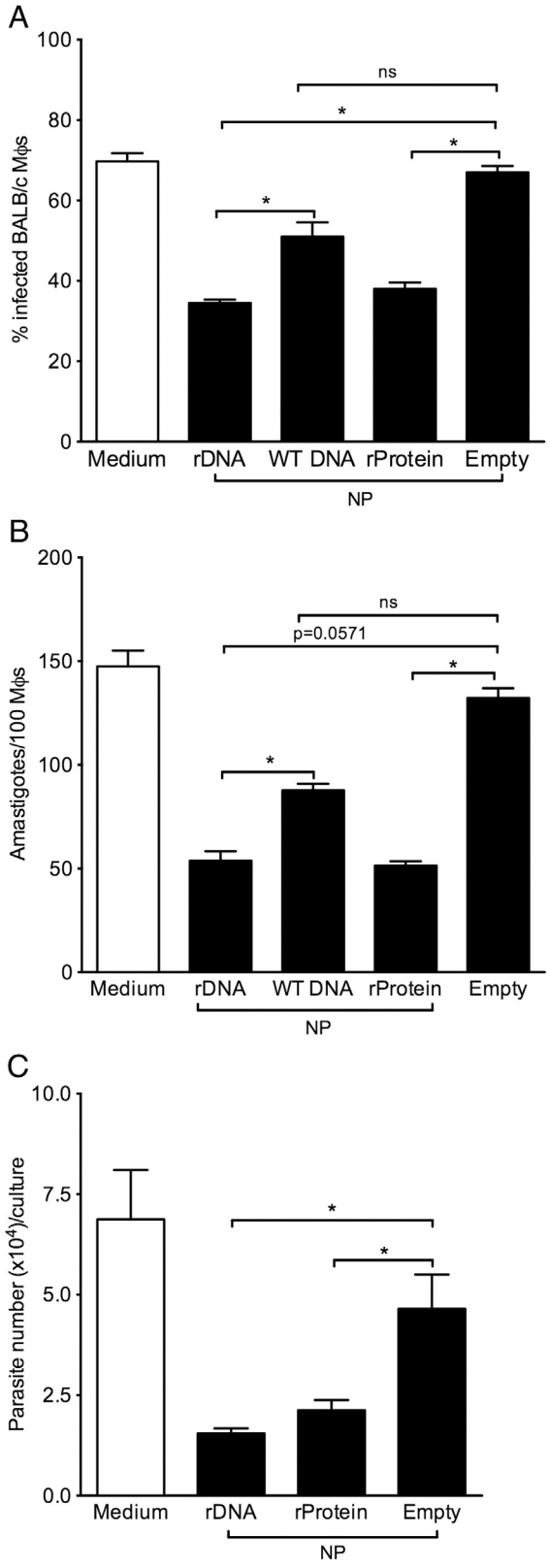
The data are presented as the mean ± SEM. The significance of the results was calculated using the following nonparametric statistical tests: two-sided Mann–Whitney for comparisons between two groups and Kruskal–Wallis followed by Dunn's multiple comparison test for comparisons between three or more groups. The analyses were conducted using GraphPad Prism 5.0 software. Differences were considered statistically significant when $P \leq 0.05$.

Results

Characterization of KMP-11-loaded PLGA nanoparticles

The nanoparticles prepared with rProtein or plasmid DNA (WT or rDNA) displayed a mean size of 300-450 nm, irrespective of the type of encapsulated antigen (WT, rDNA or rProtein) (Table 1). The mean zeta potential values were between 20 and 30 mV, indicating a positive charge at pH 7.4, which was also independent of the nanoparticle load (rDNA or rProtein). Antigen content varied from 3.5 to 7.2 μg/mg NP and encapsulation efficiency was above 43%. The presence of DOTAP was necessary for the optimal (70%-90%) encapsulation efficiency of DNA.[20] We maintained the cationic lipid in the formulation of rProtein-loaded nanoparticles, despite the decreased encapsulation of recombinant KMP-11 by approximately 50%, to allow comparisons among the different formulations. Moreover, the modulation of multiple activation pathways with DOTAP and other cationic lipids has been previously reported.[26]

The electrophoretic mobility of recombinant protein extracted from rProtein nanoparticles showed a similar profile to that obtained with recombinant KMP-11 alone (Figure 1, *A*). The absence of bands of lower molecular weight also indicates the maintenance of protein integrity after encapsulation. The mobility of naked pcDNA3-KMP-11 was similar to that observed with plasmid DNA extracted from rDNA-loaded nanoparticles and the presence of supercoiled DNA within rDNA nanoparticles



indicated structural integrity (Figure 1, *B*). We also determined the cumulative release of rDNA and rProtein from recombinant nanoparticles. The particles presented a low burst effect: 15% each of rProtein and rDNA (Figure 1, *C*) were released during the first 3 h, corresponding with the fraction located near the particle surface. Subsequently, we observed a phase of sustained release with an accumulation of ~50% (rProtein) and ~65% (rDNA) released by the time of the final observation at day 7.

KMP11-loaded PLGA nanoparticles induce parasite killing in infected macrophages

Macrophage stimulation with rDNA- and rProtein-loaded nanoparticles or with empty nanoparticles did not alter cell viability, indicating that formulations are not toxic (Supplemental Material, Fig. 2). To probe for microbicidal effects, the cells were incubated with rDNA-, WT DNA-, or rProtein-loaded nanoparticles or with unloaded (empty) nanoparticles and were subsequently infected with *L. braziliensis*. Stimulation with rDNA-loaded nanoparticles significantly decreased the number of infected macrophages when compared with WT DNA-loaded or control (empty) nanoparticles (Figure 2, *A*). Similarly, stimulation with rProtein-loaded nanoparticles also significantly decreased the parasite load when compared with control (empty) nanoparticles (Figure 2, *A*). Importantly, stimulation with recombinant KMP-11 alone failed to decrease the parasite load (Supplemental Material, Fig. 3). The average number of infected macrophages in unstimulated cultures (medium) was significantly higher [69.7 ± 4.1 (mean ± SEM)] than that of cultures stimulated with rDNA-loaded nanoparticles [34.5 ±1.7(mean ± SEM)] or with rProtein-loaded nanoparticles [(38 ±3.1) (mean ± SEM)]. Macrophage treatment with rDNA- or rProtein-loaded nanoparticles also significantly decreased the number of amastigotes per infected cell, compared to empty nanoparticles (Figure 2, *B*). Furthermore, macrophage stimulation with rDNA- or rProtein-loaded nanoparticles significantly inhibited the in vitro differentiation of *L. braziliensis* promastigotes compared to control (empty) nanoparticles (Figure 2, *C*).

KMP-11-loaded PLGA nanoparticles induce nitric oxide and superoxide production

Stimulation with rDNA- and WT DNA-loaded nanoparticles significantly increased nitric oxide (NO) production compared with control (empty) nanoparticles (Figure 3, *A*). Production of NO was lower upon treatment with rProtein-loaded or with empty nanoparticle stimulation. We detected increased superoxide levels in the cells treated with either rDNA- or rProtein-loaded nanoparticles. In these conditions, the mean fluorescence

Figure 2. Pre-stimulation of macrophages with KMP11-loaded nanoparticles (NP) promotes L. braziliensis killing. Thyoglycolate-elicited macrophages were stimulated with rDNA-, WT DNA-, or rProtein-loaded nanoparticles or with control (empty) nanoparticles. The control cultures were left unstimulated (medium). The macrophages were infected with *L. braziliensis* and assessed for the percentage of infected cells **(A)** and for the number of amastigotes per infected macrophage **(B)** using light microscopy or culture in Schneider medium **(C)**. The data are from two independent experiments and are shown as the mean ± SEM. $*P < 0.05$.

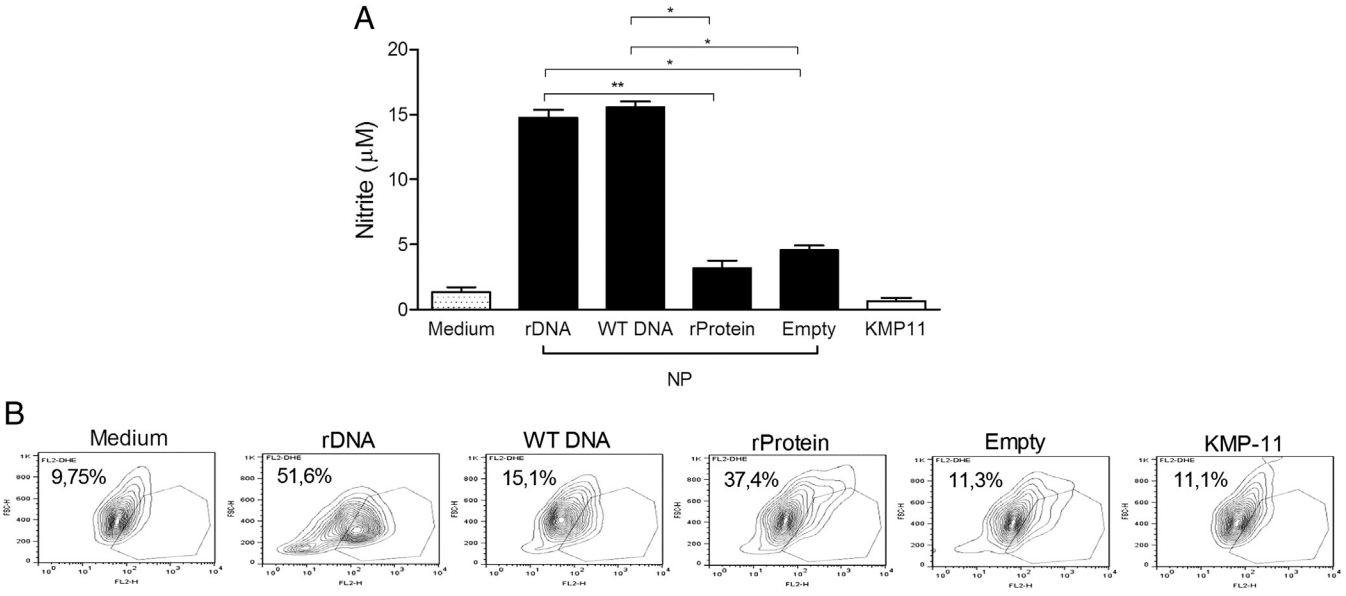


Figure 3. NO and superoxide production following macrophage stimulation with KMP11-loaded nanoparticles. Thyoglycolate-elicited macrophages were stimulated with rDNA-, WT DNA or rProtein-loaded nanoparticles or with control (empty) nanoparticles in the presence of LPS. The control cultures were left unstimulated (medium). **(A)** After 24 h, the supernatants were assayed for nitrite production. The data are from a single experiment representative of two independent experiments and are shown as the mean ± SEM. $^{**}P < 0.01$. **(B)** The cells were incubated with 10 μM DHE and analyzed for superoxide production by flow cytometry. The data are shown as the percentage of DHE^{+} cells. The results shown are from one experiment representative of two independent experiments.

intensity, as detected by flow cytometry, was higher than that of the control cultures (Figure 3, *B*), including cells stimulated with WT DNA-loaded and empty nanoparticles. Therefore, we may associate parasite killing upon stimulation with KMP-11-loaded nanoparticles with induction of the oxidative burst.

KMP-11-loaded PLGA nanoparticles promote cytokine secretion and cell recruitment

Next, we evaluated the cytokine production upon exposure to KMP-11-loaded nanoparticles. Macrophages incubated with rDNA- or rProtein-loaded nanoparticles produced significantly more TNF-α (Figure 4, *A*) and IL-6 (Figure 4, *B*) than those incubated with control (empty) nanoparticles. The production of IL-10 did not change significantly (Figure 4, *C*). When used at concentrations (3 μg/mL) similar to those of rProtein-loaded nanoparticles, recombinant KMP-11 alone failed to alter the production of TNF-α and IL-6 (Supplemental Material, Fig. 4). Macrophage stimulation with rDNA- or rProtein-loaded nanoparticles also significantly augmented CCL2/MCP-1 (Figure 5, *A*) and CXCL1/KC (Figure 5, *B*) secretion, when compared with control (empty) nanoparticles.

The biological effects of CCL2/MCP-1 and CXCL1/KC on macrophage and neutrophil recruitment were evaluated in a chemotaxis assay. The supernatants from cells stimulated with rProtein-loaded nanoparticles promoted macrophage recruitment compared with control supernatants (Figure 5, *C*), indicating the presence of bioactive CCL2/MCP-1 (Figure 5, *A*). In parallel, the supernatants from cells stimulated with either rDNA NP- or rProtein-loaded nanoparticles also significantly increased neutrophil recruitment in comparison with the control supernatants (Figure 5, *D*), confirming the presence of bioactive CXCL1/KC (Figure 5, *B*).

KMP-11-loaded PLGA nanoparticles trigger inflammasome activation

The uptake of poly(lactide-co-glycolide) (PLG) and polystyrene microparticles promotes inflammasome activation[27]; therefore, we also analyzed caspase-1 activation and the secretion of IL-1β and IL-18. Stimulation with either rDNA- or rProtein-loaded nanoparticles significantly increased caspase-1 activation in the presence of LPS (Figure 6, *A*) in comparison with control (empty) nanoparticles. Additionally, the secretion of mature IL-1β (Figure 6, *B*, open bars) was also elevated when compared with control (empty) nanoparticles, indicative of caspase-1 activation (Figure 6, *B*, open bars). On the contrary, the presence of the caspase-1 inhibitor Z-WEHD-FMK (Figure 6, *B*, closed bars) significantly blocked the secretion of mature IL-1β by cells stimulated with either rDNA- or rProtein-loaded nanoparticles (Figure 6, *B*, closed bars). Moreover, only rDNA-loaded nanoparticles led to increased IL-18 production when compared with control (empty) nanoparticles (Figure 6, *C*).

Because pre-stimulation with recombinant nanoparticles induced parasite killing (Figure 2) and inflammasome assembly (Figure 6, *A-C*), we next determined whether this pathway is involved in nanoparticle-induced *L. braziliensis* killing. The macrophages were pretreated with Z-WEHD-FMK, stimulated with recombinant nanoparticles and infected with *L. braziliensis*. Notably, pretreatment with the caspase-1 inhibitor significantly augmented the number of infected cells (Figure 6, *D*) and

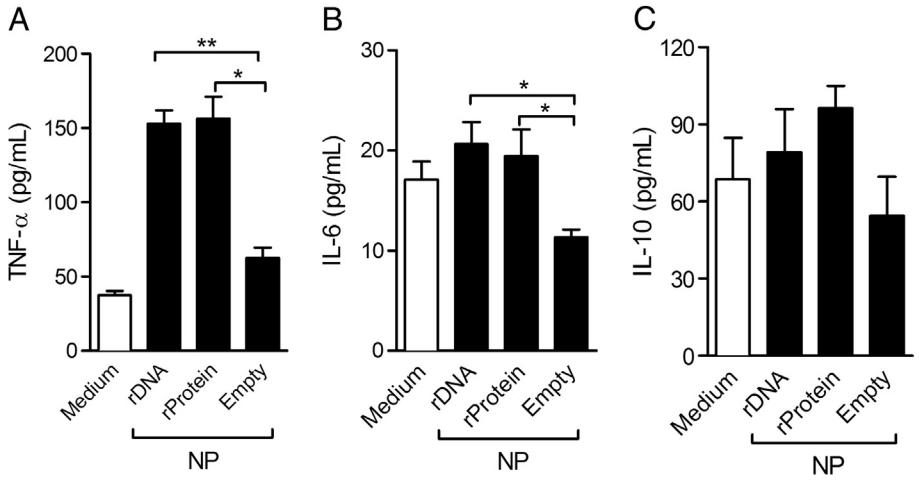


Figure 4. Cytokine secretion following macrophage stimulation with KMP11-loaded nanoparticles. Thyoglycolate-elicited macrophages were stimulated with rDNA- or rProtein-loaded nanoparticles or with control (empty) nanoparticles. The control cultures were left unstimulated (medium). The presence of secreted TNF-α **(A)**, IL-6 **(B)** and IL-10 **(C)** were detected in culture supernatants using a Cytometric Bead Array. The data are from a single experiment representative of three independent experiments and are shown as the mean ± SEM. $*P < 0.05$ and $**P < 0.01$.

amastigotes (Figure 6, *E*), despite pre-stimulation with rDNA- or rProtein-loaded nanoparticles. This effect was not observed in cells stimulated with unloaded (empty) nanoparticles.

Discussion

PLGA is a biodegradable and biocompatible polymer used for the preparation of microparticles and nanoparticles. The uptake of antigen-loaded PLGA particles by dendritic cells enhanced antigen delivery,[28] prolonged MHC class-I presentation[29] and promoted a Th1 immune response.[30] We showed that immunization with PLGA nanoparticles loaded with KMP-11, a *Leishmania* vaccine candidate,[15,17] elicited a cellular immune response in mice and led to a lower parasite load following challenge with *L. braziliensis* parasites.[12] Given these results, we studied the effects of PLGA nanoparticles loaded with KMP-11 on the innate immune response, focusing on the macrophage given its dual role as host and effector cell during *Leishmania* infection.

The stimulation of macrophages with recombinant nanoparticles followed by *L. braziliensis* infection decreased parasite load and significantly increased NO production, a hallmark of microbicidal function. Immunostimulatory CpG motifs, present in the parasite gene insert coding for KMP-11 and in plasmid DNA, can enhance phagocytic activity when presented with other stimulatory elements[31] and may account for the elevated NO production, an effect not observed with recombinant protein, for example. Differently from NO, superoxide production was higher only in cells stimulated with rDNA and with rProtein-loaded nanoparticles. Since superoxide is important for experimental *L. braziliensis* elimination,[32] this finding may explain the reduced parasite load observed in these conditions. Internalization of silver nanoparticles by monocytes leads to leakage of cathepsins into the cytoplasm, resulting in production of ROS[33] and PLGA nanoparticles co-localize to endosomal compartments upon incubation with J774 macrophages.[34] In agreement, we also noted the accumulation of fluorescently labeled nanoparticles within stimulated macrophages (data not shown) suggesting that superoxide production may have resulted from internalization of the recombinant formulations.

PLGA microspheres loaded with *Histoplasma capsulatum* antigen and $LTB_{(4)}$ induced TNF-α and NO production by bone marrow-derived macrophages.[35] TNF-α is a pleiotropic inflammatory cytokine that synergizes with IFN-γ to induce cell activation and *Leishmania* killing,[36,37] dependent on induction of NO[38] and, in the case of *L. braziliensis*, of superoxide.[32] In this study, stimulation of macrophages with rDNA- or rProtein-loaded nanoparticles induced production of TNF-α, IL-6 and superoxide, and we propose that these mediators ultimately synergized toward *L. braziliensis* killing. Because KMP-11 alone, not loaded into nanoparticles, promoted the secretion of TNF-α, IL-6 and CCL2/MCP-1 only when employed at high concentrations (≥ 15 μg/mL) (Supplemental Material, Fig. 3), we suggest that the entrapment of KMP-11 in PLGA nanoparticles significantly enhances the ability of KMP-11 to induce parasite killing.

Chemokines are major players that regulate the sequential steps of leukocyte rolling, firm adherence, and transmigration to sites of inflammation. CXCL1 is a dominant chemokine in murine inflammatory responses[39] and is critical for neutrophil recruitment.[40] CCL2 induces chemotaxis, the respiratory burst in human monocytes[41] and stimulates the elimination of *Leishmania* parasites..[42] CCL2 and CXCL1 were detected in the supernatants of cells stimulated with KMP-11-loaded nanoparticles, suggesting that CCL2 may have synergized with superoxide in the killing of *L. braziliensis*. Following immunization with KMP-11-loaded nanoparticles, we observed an intense inflammatory reaction at the inoculation site (D.M Santos, unpublished), indicating in vivo effects of cellular recruitment and inflammation.

Inflammasomes are multiprotein complexes containing one or more nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) that assemble in response to danger signals. Among these, the Nalp3 inflammasome is activated by "danger-

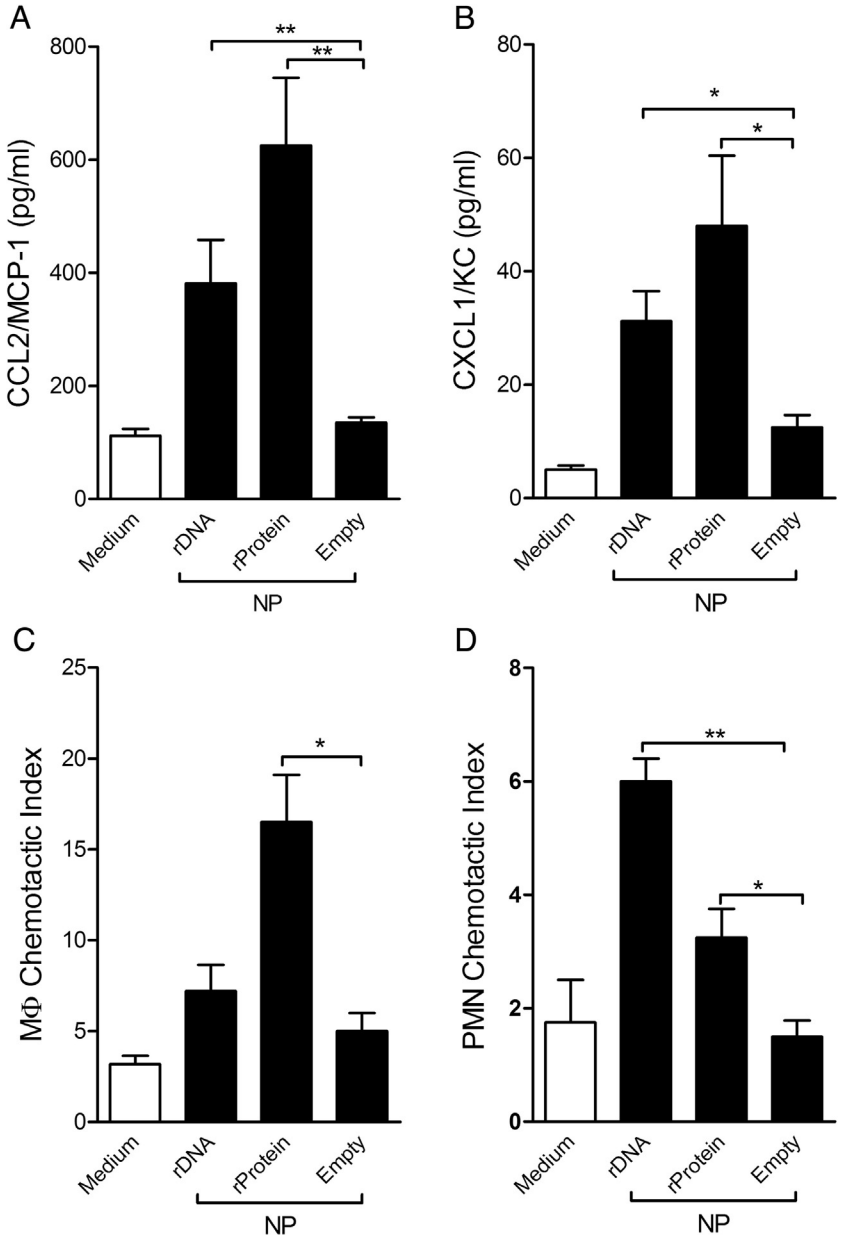


Figure 5. Chemokine production and cell chemotaxis following macrophage stimulation with KMP11-loaded nanoparticles. Thyoglycolate-elicited macrophages were stimulated with rDNA- or rProtein-loaded nanoparticles or with control (empty) nanoparticles. Control cultures were left unstimulated (medium). CCL2/MCP-1 **(A)** and CXCL1/KC **(B)** were detected using a Cytometric Bead Array and an ELISA, respectively. The culture supernatants were assayed for macrophage **(C)** and neutrophil **(D)** chemotaxis. The data are from a single experiment representative of two independent experiments and are shown as the mean ± SEM. *$P < 0.05$ and ** $P < 0.01$.

associated molecular patterns" or DAMPs (non-microbial/non-pathogenic), such as ATP,[43] uric acid crystals[44] and aluminum salt particles.[45] Stimulation of Nalp3 regulates caspase-1 activity, leading to the cleavage of pro-IL-1β and pro-IL-18 into the bioactive cytokines IL-1β and IL-18. Indeed, PLGA microparticle uptake by DCs promotes IL-1β secretion and caspase-1 activation[27] and carbon nanotubes also exert this effect in human monocytes.[46] Lastly, phagocytosis of particulate structures leads to lysosomal rupture, release of cathepsin B into the cytoplasm and inflammasome activation.[47] Here, co-stimulation with KMP-11-loaded nanoparticles and LPS activated caspase-1, leading to secretion of IL-1β and IL-18, indicative of inflammasome triggering. We may suggest that nanoparticle internalization led to superoxide production and inflammasome triggering, culminating in parasite killing. Of note, IL-18 secretion was detected only upon stimulation with rDNA-loaded nanoparticles. Two recent studies showed that, in response to intracytoplasmic nucleic acids, the AIM2 inflammasome regulates the caspase-1-dependent production of bioactive IL-1β and IL-18.[48,49] Mice deficient in *Aim2* and *Asc* showed reduced IL-18 concentrations when challenged with mCMV.[50] We then propose that stimulation with rDNA-loaded

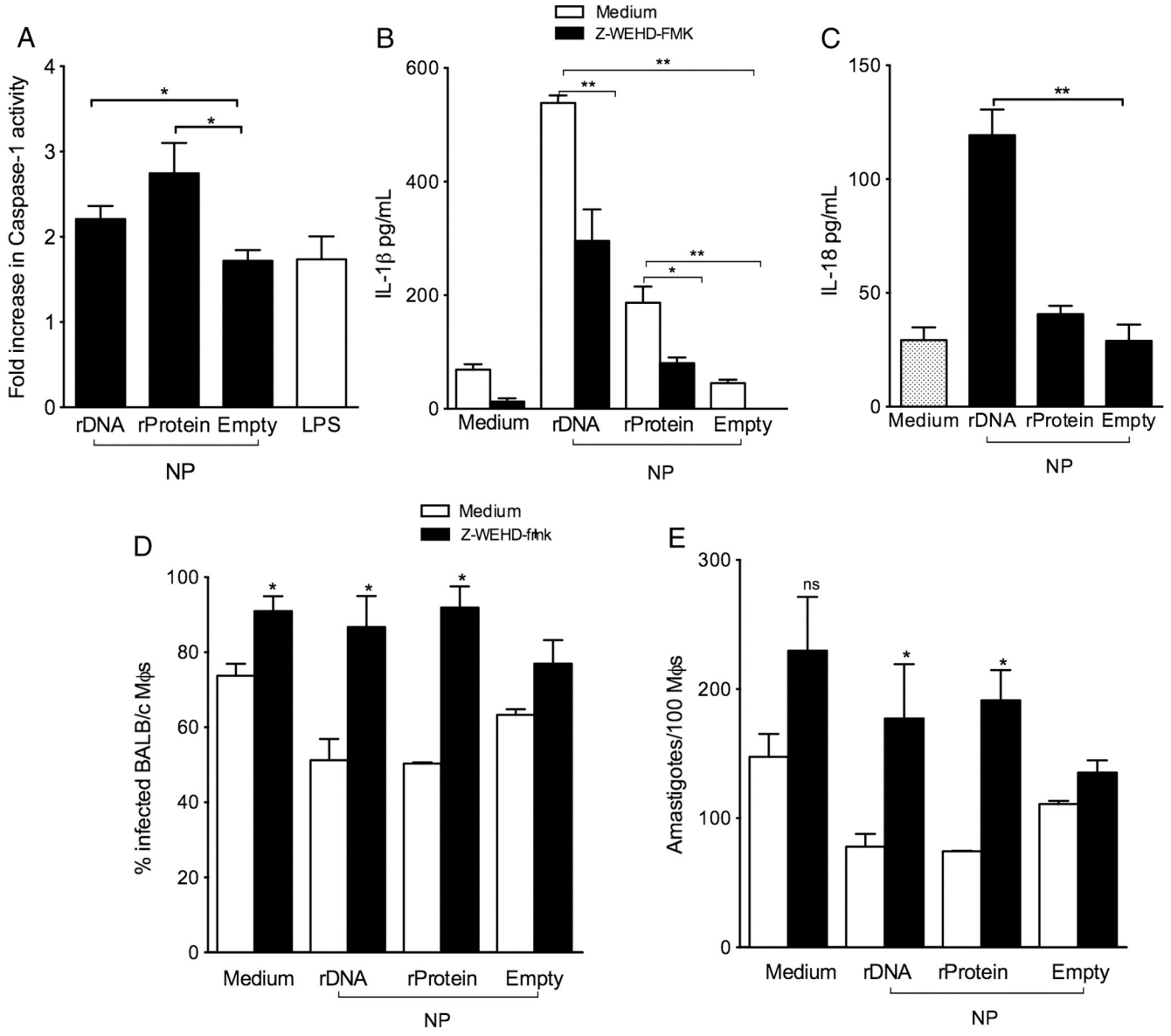


Figure 6. Activation of the inflammasome following stimulation of macrophages with KMP11-loaded nanoparticles. Thyoglycolate-elicited macrophages were stimulated with rDNA- or rProtein-loaded nanoparticles or with control (empty) nanoparticles in the presence of LPS. Caspase-1 activity **(A)** was detected using a colorimetric assay. The data are represented as the fold increase in the caspase-1 activity over that of cells incubated in medium only. **(B)** Cells were stimulated as described in the presence (closed bars) or absence (open bars) of Z-WEHD-FMK. Secreted IL-1β was detected using an ELISA. **(C)** The cells were stimulated as described above, and IL-18 was detected using an ELISA. **(D)** Cells were treated with Z-WEHD-FMK (closed bars) or were untreated (open bars) and were subsequently stimulated with rDNA- or rProtein-loaded nanoparticles or with control (empty) nanoparticles. The cells were infected with *L. braziliensis* and assessed for the percentage of infected macrophages and for the number of amastigotes per infected macrophage **(E)** using light microscopy. The data are shown as the mean ± SEM and originate from two (A and C-E) or three **(B)** independent experiments. $*P < 0.05$ and $**P < 0.01$.

nanoparticles may also trigger the AIM2 inflammasome, in parallel with the Nalp3 sensor.

PLGA nanoparticles loaded with a *Leishmania* protein (KMP-11) promote a powerful innate immune response in macrophages, characterized by the secretion of pro-inflammatory cytokines and chemokines, superoxide production and also inflammasome triggering. Collectively, these effects lead to cell activation and *L. braziliensis* killing. These results build on our previous findings in which immunization with KMP-11-loaded nanoparticles induced an immune response in mice and a reduction in parasite load, following a challenge with live parasites.[12] Based on this evidence, we propose that the current formulations can be further pursued as delivery vehicles in the development of vaccines against cutaneous leishmaniasis caused by *L. braziliensis*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2013.04.003.

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