There are currently no effective vaccines for visceral leishmaniasis, the second most deadly parasitic infection in the world. Here, we describe a novel whole-cell vaccine approach using *Leishmania infantum chagasi* promastigotes treated with the psoralen compound amotosalen (S-59) and low doses of UV A radiation. This treatment generates permanent, covalent DNA cross-links within parasites and results in *Leishmania* organisms termed killed but metabolically active (KBMA). In this report, we characterize the *in vitro* growth characteristics of both KBMA *L. major* and KBMA *L. infantum chagasi*. Concentrations of S-59 that generate optimally attenuated parasites were identified. Like live *L. infantum chagasi*, KBMA *L. infantum chagasi* parasites were able to initially enter liver cells *in vivo* after intravenious infection. However, whereas live *L. infantum chagasi* infection leads to hepatosplenomegaly in mice after 6 months, KBMA *L. infantum chagasi* parasites were undetectable in the organs of mice at this time point. *In vitro*, KBMA *L. infantum chagasi* retained the ability to enter macrophages and induce nitric oxide production. These characteristics of KBMA *L. infantum chagasi* correlated with the ability to prophylactically protect mice via subcutaneous vaccination at levels similar to vaccination with live, virulent organisms. Splenocytes from mice vaccinated with either live *L. infantum chagasi* or KBMA *L. infantum chagasi* displayed similar cytokine patterns *in vitro*. These results suggest that KBMA technology is a potentially safe and effective novel vaccine strategy against the intracellular protozoan *L. infantum chagasi*. This approach may represent a new method for whole-cell vaccination against other complex intracellular pathogens.

*Leishmania* species infect 2 million humans annually, and 70 thousand die from the visceral form of leishmaniasis every year (28). Transmission of *Leishmania* occurs following the bite of a parasite-bearing phlebotomine sandfly, followed by uptake of infectious metacyclic promastigotes by neutrophils and macrophages. Various disease manifestations, which depend upon the species of infecting parasite and the genetics of the host (5), range from self-resolving cutaneous lesions to potentially fatal visceral infection of the liver, spleen, and bone marrow (48). Resolution of infection is associated with the induction of macrophage nitric oxide (NO) (dependent upon inducible nitric oxide synthase [iNOS]), gamma interferon (IFN-γ), reactive oxygen species (ROS), and specific CD4+ type 1 helper (Th1) and CD8+ lymphocyte responses (25, 38). *Leishmania* species employ distinct mechanisms to elude effector arms of the immune system, including inhibition of NO- and ROS-mediated macrophage killing and phagolysosomal fusion, inhibition of cell-mediated immunity via blocking of antigen presentation and cytokine production, and recruitment of regulatory T cells or other interleukin-10 (IL-10)-producing cells (6, 16, 30, 36).

Recent evidence supports the contention that sustained immunity may require persistent low-level infection with parasites (2, 47). In some areas of endemicity, inoculation of live *Leishmania major* parasites in the skin of at-risk individuals with the goal of inducing a mild infection and subsequent immunity is performed (43, 44). However, this practice, termed leishmanization, has been largely abandoned as unsafe as the vaccinating parasite retains virulence and induces serious pathology at significant frequencies. To overcome this problem, investigators are exploring the use of genetically modified organisms that retain vaccinating potential but lack the ability to reactivate or cause disease pathology (35, 39, 40, 46). Additional vaccination strategies in humans have included heat-killed and parasite subunit vaccination along with adjuvants such as *Mycobacterium bovis* BCG, but none has resulted in an effective prophylactic vaccine (4, 10, 14, 18, 22, 23, 29).

Recently, our collaborators have developed a technology for killing organisms in blood products by treatment with micromolar quantities of amotosalen hydrochloride (S-59), a DNA cross-linking psoralen (12). Treatment with S-59, followed by UV type A (UVA) light photoactivation, induces DNA monoadducts and interstrand cross-links which block DNA replication and transcription by preventing strand separation, resulting in mitotic arrest and eventual cell death (49). DNA adduct number and pathogen inactivation efficiency are directly related to S-59 concentration. This process effectively kills multiple pathogens contaminating blood products, including HIV, *Plasmodium falciparum*, and *Leishmania*, and is widely used to sterilize blood products for human use (15, 26).

This same technology has also been applied as a strategy to attenuate bacterial organisms so that they may safely be utilized as vaccines. The first microbe successfully attenuated was recombi-
nant Listeria monocytogenes. When treated with nanomolar doses of S-59 (plus UVA), Listeria bacteria were rendered replication incompetent but remained metabolically active (8). This resulted in Listeria that retained the ability to express heterologous antigens of vaccine interest, invade mammalian cells, and stimulate potent adaptive cellular immune responses. Similarly, psoralen treatment of Bacillus anthracis and Salmonella enterica serovar Typhimurium strains also resulted in replication-incompetent populations capable of inducing protective immunity in small-animal models (24, 41). We hypothesized that this approach, termed killed but metabolically active (KBMA), might also have utility in the intracellular, macrophage-tropic pathogen Leishmania. Here, we describe the development of KBMA Leishmania species associated with both cutaneous (L. major) and visceral (Leishmania infantum chagasi) forms of leishmaniasis and demonstrate the feasibility of this novel strategy of whole-cell vaccination against eukaryotic pathogens.

MATERIALS AND METHODS

Animals and parasites. Six- to eight-week-old female BALB/c mice (Jackson Laboratories) were used throughout. All experiments were approved by the Institutional Animal Care and Use Committee at Los Angeles Biomedical Research Institute. Firefly luciferase-expressing L. major (L. major-luciferase) promastigotes, derived from Friedlin (MHOM/IL/80/ Friedlin) clone V1, were engineered to express luciferase following integration of the construct pIRISAT-LUC-TK (B5113) into the rRNA locus (45; also S. M. Hickerson and S. M. Belevy, unpublished data). These L. major-luciferase parasites were routinely maintained at 26°C in M199 (powder; Sigma) medium supplemented with 1 M HEPES (pH 7.4), 10 mM adenine, 0.1% BSA, 0.25% hemin, 100 IU penicillin, 100 mg/ml streptomycin, 10% fetal bovine serum, and 0.25 mg/ml gentamicin. The L. infantum chagasi strain MHOM/BR/00/1669 (previously known as Leishmania infantum [27]) was originally isolated from a patient with visceral leishmaniasis in northeast Brazil and was maintained by serial intracardiac injection in outbred male golden hamsters. Hemolagellate minimal essential medium (HOMEM) was prepared as previously described (3). Promastigotes were cultured in HOMEM at 26°C and passaged for no more than 4 weeks before use in experiments. KBMA Leishmania parasites were prepared by incubating stationary-phase promastigotes in 100 nM amotosalen (S-59) for 1 h, followed by cross-linking of DNA with 5.4 J/cm² UVA irradiation in an Intrepid Illuminator (Cerus Corporation). Heat-killed organisms were prepared by incubation in a 100°C bath for 10 min. For carboxyfluorescein succinimidyl ester (CFSE)-based experiments, L. infantum chagasi promastigotes were stained with 5 µM CFSE for 10 min, washed three times with phosphate-buffered saline (PBS), and then cultured in HOMEM for the duration of in vitro experiments or resuspended in sterile saline for in vivo experiments.

In vitro assays of parasite function. Parasite luciferase activity was measured using a Luciferase Assay System (Promega) by sampling cultures daily, lysing with 5× Reporter Lysis Buffer (Promega), and promptly measuring relative luminescence units (RLU) after luciferin addition using a Sirius Single Tube Luminometer (Berthold Detection Systems). In vitro macrophage infection experiments were performed using murine bone marrow-derived macrophages (BMDM) as described previously (11), which were cocultured with promastigotes at a multiplicity of infection (MOI) of 20 for 1 h before cultures were washed. Infection levels were quantitated 48 h later by microscopy of Giemsa-stained cells. Macrophage killing function, as measured by induction of nitric oxide production, was determined by infecting BMDM with promastigotes. To overcome Leishmania-induced macrophage suppression and thus induce nitric oxide secretion, medium was supplemented with 10 units/ml recombinant murine IFN-γ. Supernatants were assayed by the Griess method as previously described (34).

L. major footpad infections and bioluminescent imaging. For all infection experiments, Leishmania promastigotes were grown to stationary phase and then washed three times in PBS and resuspended in sterile saline for injection into animals. Luciferase-expressing L. major promastigotes were injected into footpads in 50-µl aliquots at various concentrations (see Fig. 1). Luciferase activity was measured by serial bioluminescent imaging at the indicated time points. Anesthetized mice were injected intraperitoneally with 100 µl of 3 mg/ml of the luciferase substrate, d-luciferin, and imaged after 15 min in an IVIS 100 Imaging System (Xenogen). Analysis of images was performed using Living Image software.

Mouse immunization and challenge with L. infantum chagasi. Live or KBMA stationary-phase promastigotes (10²/100 µl of saline) were subcutaneously injected into the shaved dorsal-cervical area. Mice were immunized three times at 2-week intervals. Two or 8 weeks after the final boost, mice were challenged intravenously with 10⁴ stationary-phase promastigotes in 200 µl of saline. These vaccination schedules and delays to challenge were chosen to be consistent with existing models and with our understanding of the development of adaptive immunity.

In vivo assay of mouse liver parenchymal infection. Twenty-four to 72 h following intravenous injection of CFSE-labeled parasites, BALB/c mice were sacrificed, and livers were dissected. Single-cell suspensions were prepared using glass slide maceration, and the homogenate was passed through nylon mesh; CFSE-positive cells were quantitated by flow cytometry on a FACS Calibur (BD) instrument.

Determination of organism parasite loads by microscopy and quantitative PCR. Vaccinated mice were euthanized 4 weeks after infectious challenge. Livers and spleens were removed from challenged mice at the indicated times and weighed. Multiple glass slide touch preparations of each organ were Giemsa stained and microscopically assessed by a blinded scorer. A minimum of 500 cells were counted for each slide. The number of parasites/organ was calculated as the number of amastigotes per cell nuclei times the organ weight (in mg) times 2 × 10³, as previously described (7, 50). To determine parasite loads by PCR, total genomic DNA was harvested from livers and spleens using an UltraClean Tissue DNA Isolation Kit (Mo Bio Laboratories). Quantitative PCR assays were then performed using a TaqMan system (Applied Biosystems), with 200 nM primers/probe and genomic DNA template diluted 1:10 following column elution. L. infantum chagasi parasite DNA and mouse DNA were detected using primers specific for GP63 and tumor necrosis factor alpha (TNF-α), respectively. Primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA) with the following sequences: GP63 for, 5′-CTA CGT CCG CTG CGA CAC CTT-3′; GP63 rev, 5′-AGC CGA GGT CTT GGA AGA T-3′; GP63 probe, 5′-35′-FAM-AGC CCG CAC CGG CCT GGT-30′-TAMsp-3′ (where FAM is 6-carboxyfluorescein and TAMsp is 6-carboxytetramethylrhodamine); TNF-α for, 5′-TCC TTC TCA TCA GTG CTA TGG CCC C-3′; TNF-α rev, 5′-CAG GCA GGA TCT ATG CAT CTTA GAC CC-3′; TNF-α probe, 5′-/56-JOEN-TGG AGG AAG GCC AGT TAG GCA TGG GA-3BHQ-2′-3′ (where JOEN is 6-carboxy-4′,5′-dichloro-2′-7′-dimethoxyfluorescein and BHQ is Black Hole quencher). GP63 threshold cycle (Ct) values below 35 were converted to absolute parasite counts using previously determined standard curves and were normalized to tissue DNA amounts by TNF-α Ct values, as previously described (9). GP63 Ct values above 35 were considered to be below the limit of detection, based on negative controls with no Leishmania DNA template.

Flow cytometry and intracellular cytokine staining. CFSE-stained parasites were sampled from culture flasks and analyzed by flow cytometry. Splenocytes from immunized animals were cultured in complete RPMI medium for 72 h in the presence of 10 units/ml of recombinant mouse IL-2 (BD) and 3 × 10⁴ live promastigotes as an antigenic stimulant. In the final 6 h of culture, medium and promastigotes were supplemented with 1 µl/ml GolgiPlug (containing brefeldin A; BD). Cells were stained with CD4-phycocerythrin (PE) (clone RM-45; BD) or CD8-PE (clone 53-6.7; BD) antibodies at a final concentration of 5 µg/ml, permeabilized using a Cytofix/Cytoperm Kit (BD) according to the manufacturer.
er’s instructions, stained intracellularly with IFN-γ–fluorescein isothiocyanate (FITC) (clone XMG1.2; BD) (final concentration of 2 μg/ml), and analyzed on a FACSCalibur flow cytometer.

Luminex multiplex cytokine analysis. Cell culture supernatants were analyzed using a mouse Th1-Th2 panel (Bio-Rad) according to the manufacturer’s instruction. Cytokines included IFN-γ, IL-12 (p70), IL-2, IL-4, IL-5, and IL-10. Samples were analyzed on a Luminex 200 instrument. Data were analyzed with BeadView software (Upstate).

Statistical methods. Experiments were analyzed by two-tailed t tests. Unless stated otherwise, a P value of <0.05 was considered significant.

RESULTS
Characterization of KBMA Leishmania. We hypothesized that species of Leishmania, a eukaryotic protozoan pathogen, could be rendered replication incompetent while retaining functional and immunogenic characteristics relevant to host infection, as previously achieved with various killed but metabolically active bacterial strains (8, 24, 41). To test this hypothesis, we utilized strains of L. major and L. infantum chagasi, agents of cutaneous and visceral leishmaniasis, respectively. Use of transgenic strains of Leishmania engineered to express the firefly luciferase gene (45) allowed us to monitor the progression of both in vivo infections and in vitro cultures of parasites. We first assessed the ability of killed but metabolically active (KBMA) parasites to establish infection in a footpad model of cutaneous leishmaniasis. Luciferase-expressing L. major promastigotes were treated with various doses of S-59 followed by UVA illumination to induce stable DNA cross-links. Increasing numbers of untreated promastigotes or a fixed number (10^7) of promastigotes treated with increasing doses of S-59 (plus UVA) were injected into the footpads of BALB/c mice, and the course of infection was followed by serial bioluminescent imaging (Fig. 1). Each footpad shown in this figure received a different inoculum or S-59 dose. Inoculation with untreated promastigotes (panel A) led to progressive footpad swelling, ulceration, and luciferase production. Infections were detectable at earlier time points postinfection with higher numbers of parasites in the initial inoculation (Fig. 1A). In comparison, inoculations of 10^7 L. major promastigotes (similar to the right-most footpad shown in panel A) treated with increasing doses of S-59 (plus UVA) were injected into the footpads of BALB/c mice, and the course of infection was followed by serial bioluminescent imaging (Fig. 1). Each footpad shown in this figure received a different inoculum or S-59 dose. Inoculation with untreated promastigotes (panel A) led to progressive footpad swelling, ulceration, and luciferase production. Infections were detectable at earlier time points postinfection with higher numbers of parasites in the initial inoculation (Fig. 1A). In comparison, inoculations of 10^7 L. major promastigotes (similar to the right-most footpad shown in panel A) treated with increasing doses of S-59 (plus UVA) all established initial infections, but doses of 100 nM S-59 and above resulted in progressive diminution of luminescence to low levels by day 8 (panel B). Promastigotes treated with only 10 nM S-59 caused a
progressive infection through day 29, which led to an ulcerating lesion by day 43. There were no signs of swelling or infection in footpads harboring \textit{L. major} treated with the higher doses of S-59 at the end of 6 weeks. These data suggest that doses of S-59 of at least 100 nM, combined with UVA irradiation, render \textit{L. major} highly attenuated, reducing the infectious equivalent by a factor of at least 10^4.

To further characterize the effects of this treatment on parasite survival, we measured protein expression levels of S-59/UVA-treated promastigote populations in liquid cultures grown over 3 weeks. Cultures of luciferase-expressing \textit{L. major} were initially seeded with various numbers of untreated promastigotes and measured to establish baseline growth curves. Doses of S-59 (plus UVA) of at least 100 nM induced a progressive decrease of metabolic activity in cultures seeded with 10^7 treated \textit{L. major} parasites. Treatment of 10^7 promastigotes with 750 nM resulted in complete inhibition of luciferase activity after 3 weeks (Fig. 2A). By comparing the growth curves of serial dilutions of untreated parasites with growth curves of 10^7 \textit{L. major} parasites treated with increasing doses of S-59, one can estimate that 500 nM and 100 nM resulted in inhibition of growth by factors of approximately 10^6 and 10^4, respectively (Fig. 2A). For example, 10 untreated parasites required the same amount of time to reach stationary phase as 10^7 \textit{L. major} promastigotes treated with 500 nM S-59. Additionally, because confluent organisms expend all the nutrients in the culture medium, downward drift of the number of RLU after 7 to 10 days at stationary phase is expected. Experiments with the visceral disease-causing species \textit{L. infantum chagasi} yielded similar results. Cultures of \textit{L. infantum chagasi} promastigotes treated with 100 nM S-59 (plus UVA) lost all detectable luciferase activity by 21 days after initial seeding (Fig. 2B). To monitor replication following S-59/UVA exposure, we stained live \textit{L. infantum chagasi} with CFSE and measured the intracellular concentration over time. Promastigotes treated with 10 nM S-59 (plus UVA) replicated only slightly more slowly than untreated controls (Fig. 2C). However, treatment with 100 nM S-59 (plus UVA) blocked nearly all cellular division. For subsequent experiments, therefore, \textit{L. infantum chagasi} promastigotes were treated with 100 nM S-59 (plus UVA); they are referred to here as KBMA \textit{L. infantum chagasi}.

KBMA \textit{L. infantum chagasi} parasites do not persist or cause organomegaly in vivo. Virulent \textit{L. infantum chagasi} parasites injected into susceptible mouse strains replicate as amastigotes in both liver and spleen, multiplying rapidly in the liver before eventual spontaneous clearance and progressing slowly in the spleen (48). To determine the ability of KBMA \textit{L. infantum chagasi} to persist long-term in vivo, we intravenously injected five BALB/c...
mice each with $10^7$ untreated live or KBMA \textit{L. infantum} \textit{chagasi} parasites. Six months after infection, all mice inoculated with live \textit{L. infantum} \textit{chagasi} displayed marked hepatosplenomegaly, while all of the KBMA \textit{L. infantum} \textit{chagasi}-inoculated mice had organ sizes similar to uninfected mice (Fig. 3). Microscopic analysis of Giemsa-stained tissue sections demonstrated liver and spleen parasite loads of $2.6 \times 10^7 \pm 0.6 \times 10^7$ and $2.0 \times 10^7 \pm 0.5 \times 10^7$ parasites per organ, respectively, in live \textit{L. infantum} \textit{chagasi}-infected mice. No parasites, however, could be visualized microscopically in any of the mice infected with KBMA \textit{L. infantum} \textit{chagasi}. Quantitative PCR is a more sensitive method of detection and demonstrated large parasite loads in the livers and spleens of live \textit{L. infantum} \textit{chagasi}-infected animals. However, we were unable to detect any \textit{Leishmania} DNA in the organs of KBMA \textit{L. infantum} \textit{chagasi}-infected animals (data not shown). Thus, while live \textit{L. infantum} \textit{chagasi} infection caused organomegaly and parasite persistence in mouse liver and spleen 6 months after infection, KBMA \textit{L. infantum} \textit{chagasi} parasites were undetectable in tissues at this time point, and KBMA \textit{L. infantum} \textit{chagasi}-infected animals showed no signs of associated organomegaly or detectable parasite presence.

\textbf{KBMA \textit{L. infantum} \textit{chagasi} parasites enter macrophages and induce NO production.} \textit{Leishmania} are capable of infecting macrophages both \textit{in vivo} and \textit{in vitro}. Entry of parasites into macrophages leads to induction of iNOS expression and secretion of NO in macrophages primed with IFN-\gamma (17). To determine if KBMA \textit{L. infantum} \textit{chagasi} parasites also induce a similar macrophage response, we exposed primary murine bone marrow-derived macrophages (BMDM) from BALB/c mice to \textit{Leishmania in vitro} and measured supernatant levels of NO. In the presence of exogenously added IFN-\gamma, KBMA \textit{L. infantum} \textit{chagasi} induced NO production at levels similar to live \textit{L. infantum} \textit{chagasi} (Fig. 4A). We performed similar infections of BMDM in the absence of exogenous IFN-\gamma to allow for the persistence of intracellular amastigotes. Both live promastigotes and KBMA \textit{L. infantum} \textit{chagasi} entered BMDM \textit{in vitro}, transformed into amastigotes that were...
visible intracellularly by microscopy, and persisted intracellularly at 48 h following infection, whereas heat-killed parasites were not detectable at this time point (Fig. 4B and C). These results suggest that KBMA L. infantum chagasi parasites are more similar to live parasites than to heat-killed parasites in their ability to stimulate significant NO production. Also, although infection rates are slightly reduced compared to that with live parasites, KBMA L. infantum chagasi parasites are able to enter macrophages and temporarily persist intracellularly as amastigotes.

KBMA L. infantum chagasi parasites enter liver cells following intravenous inoculation. To determine if KBMA L. infantum chagasi parasites are able to infect macrophages in vivo, promastigotes were stained with CFSE and injected intravenously into BALB/c mice. Both live and S-59/UVa-treated parasites were detected by flow cytometry in single-cell suspensions of livers from mice 24 h following inoculation, whereas heat-killed organisms were not readily detectable at this time point (Fig. 4D). The size of the CFSE-positive events as determined by forward scatter indicated that Leishmania parasites were associated with host cells (data not shown). Infected cells (associated with both live parasites and KBMA L. infantum chagasi) were detectable at 72 h following infection as well (data not shown).

Prophylactic vaccination with KBMA L. infantum chagasi protects against intravenous challenge with virulent L. infantum chagasi. To determine the protective efficacy of KBMA L. infantum chagasi vaccination, we performed vaccine/challenge experiments in susceptible BALB/c mice. High-dose live L. infantum chagasi injected subcutaneously in the dorsal neck has previously been shown to partially protect mice against subsequent L. infantum chagasi intravenous challenge (42). In our studies, animals were injected subcutaneously with 10⁷ live L. infantum chagasi or KBMA L. infantum chagasi parasites or with saline and then boosted with the same treatments twice more at 2-week intervals before intravenous challenge with live, virulent L. infantum chagasi 2 weeks after the final immunization. Animals vaccinated with KBMA L. infantum chagasi showed protection levels in the liver similar to those of mice vaccinated with live L. infantum chagasi (Fig. 5A). To determine whether vaccination effects were long lasting, we repeated the experiment with 8 weeks between the last vaccination and the infectious challenge (Fig. 5B). In both experiments, control mice that were vaccinated subcutaneously with virulent L. infantum chagasi or KBMA L. infantum chagasi, but not challenged intravenously with virulent parasites, had no detectable parasites in the touch preparations after 1 month (data not shown). Significant protection from virulent Leishmania challenge was induced by live and KBMA L. infantum chagasi vaccination regardless of the length of time after the last vaccination boost.

Whole-cell KBMA vaccination induces adaptive CD4 immune responses. To determine if vaccination with KBMA L. infantum chagasi could induce adaptive immune responses against Leishmania antigens, we measured T cell responses in the spleens of vaccinated animals. Two weeks following vaccination as above, groups of mice were sacrificed, and splenic T cells were restimulated in vitro with live L. infantum chagasi for 3 days prior to measuring intracellular IFN-γ levels. Mice in both live L. infantum chagasi (3 of 3 animals) and KBMA L. infantum chagasi (2 of 3 animals) groups demonstrated Leishmania-specific T cell IFN-γ responses above control mice (saline vaccinated) following restimulation (Fig. 6A) although these differences were not statistically significant. All Leishmania-specific IFN-γ-positive T cells detected after this 3-day antigen exposure were also positive for the surface marker CD4 but not for CD8 (data not shown). Using the same cultured splenocytes from these groups of vaccinated mice, we analyzed supernatants from Leishmania-restimulated splenocytes for specific cytokines, using a multiplex cytometric bead array. Splenocytes from animals vaccinated with either live or KBMA L. infantum chagasi showed elevated mixed cytokine responses over saline-vaccinated animals (Fig. 6B). Splenocytes from vaccinated animals demonstrated higher IFN-γ secretion only when restimulated with Leishmania ex vivo. Overall, these findings suggest that vaccination with KBMA L. infantum chagasi can induce Leishmania-specific protective immune responses that are functionally similar to those induced by live, fully virulent Leishmania.

DISCUSSION

Successful vaccination with live, whole-cell organisms depends upon safe, reliable attenuation coupled with retention of immunogenicity. Several strategies to achieve this balance exist. Killed pathogens alone often do not elicit long-lasting immunity, likely due to their inability to access certain cellular compartments or express virulence-associated genes. Some targeted gene deletion strategies utilize live organisms that are nonpathogenic due to the lack of defined virulence factors (35, 39, 40, 46), but these strains can theoretically be prone to reversion to pathogenicity in vivo. Here, we apply a novel method of attenuation to the eukaryotic pathogen Leishmania, rendering the parasite unable to replicate. However, KBMA parasites maintain functionality comparable to live parasites during early stages of infection, before a progressive diminution in metabolic activity. We demonstrate that L. infantum chagasi parasites treated with S-59 psoralen and UVA irradiation are capable of entering macrophages both in vitro and in vivo, are able to make the complex life cycle transition from promastigote to amastigote form, and can activate macrophages to produce microbicidal nitric oxide. When given as a subcutaneous vaccine, KBMA L. infantum chagasi partially protects susceptible
BALB/c mice from a virulent challenge of *L. infantum chagasi* to a similar degree as live parasites. Both types of organisms elicit splenocytes that display similar cytokine profiles in vitro. Importantly, unlike live *L. infantum chagasi*, KBMA *L. infantum chagasi* parasites are avirulent and do not result in organomegaly or disseminated infection when introduced intravenously in mice.

The ability to grow *Leishmania* promastigotes in culture allowed us to directly assess the effects of KBMA treatment on in vitro growth characteristics of two different *Leishmania* species. The dose of S-59 (plus UVA) that inhibited luciferase expression by day 21 in *L. infantum chagasi* (100 nM) did not completely prevent outgrowth of a similar culture of *L. major* (Fig. 2A and B). An identical dose of S-59 (plus UVA) significantly blocked proliferation of *L. infantum chagasi* as measured by CFSE diminution experiments (Fig. 2C). Although these in vitro assays suggest levels of attenuation slightly less than 100% inactivation, similarly treated KBMA *Leishmania* species were not detected in vivo weeks or months following inoculation in either a cutaneous model (Fig. 1) or a visceral model (Fig. 3) of murine infection. Our results suggest that significant levels of attenuation have been achieved to prevent functional virulence and to justify additional research in animal models. As shown in Fig. 3, KBMA attenuation results in a significantly attenuated whole-cell vaccine. However, additional safety measures should be explored as this approach is studied further. Previous successful generation of KBMA bacterial species, including *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, and *Bacillus anthracis* (8, 24, 41), have incorporated inactivation of nucleotide excision repair genes. Thus, these genes could be targeted in the future for additional safety advantages with little impact on immunogenicity.

There are several important differences between animal models of leishmaniasis and human disease. One important similarity, especially with respect to cutaneous lesions, is that resolution of an infection with live parasites results in lasting immunity. In contrast, inoculation with heat-killed *Leishmania* alone does not generally elicit long-lasting protection; in fact, heat-killed *L. major* vaccination can actually be immunosuppressive when used in mice previously immune to *Leishmania* (32). Heat-killed or protein preparations of *Leishmania* can be made more immunogenic by the inclusion of adjuvant molecules, and repeated inoculations of killed parasites can also generate anti-*Leishmania* immunity (31). However, there are likely intrinsic differences between the ways live parasites and killed parasites interact with the host to determine subsequent immunity. The data presented here demonstrate that S-59/UVA-treated promastigotes, which are significantly attenuated by several measures in vitro and in vivo, have similar protective and immunostimulatory profiles as live, virulent promastigotes. Both live and KBMA *L. infantum chagasi* vaccination induces a mixed cytokine response including the Th1-associated cytokines IFN-γ and IL-12, as well as the Th2-
associated cytokines IL-4, IL-5, and IL-10. This combination of type 1 and type 2 cytokines may represent an initial in vivo immune response that allows parasites to briefly persist; however, this initial analysis of cytokine expression was carried out at a single time point and is far from comprehensive. It does nonetheless suggest qualitative similarities between live and KBMA immunization that will be interesting to more fully characterize in future studies.

The vaccination used as a positive control in visceral leishmaniasis models in mice is a high-dose, subcutaneous inoculation of live, virulent promastigotes. This approach does not lead to disseminated parasites but confers a partial level of protection against subsequent intravenous challenge. Leishmania promastigotes rendered avirulent, either by long-term cultivation or by genetic deletion of the dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene locus, do not confer protection by this route (42). The fact that KBMA organisms retain some characteristics associated with virulence, especially during early infection, may allow for increased levels of persistence and possibly allow for prolonged antigen presentation. Durable immunity to reinfecion with L. major depends upon persisting parasites in the skin, which are not completely cleared by the host, in part due to IL-10-producing CD4+ CD25+ regulatory T cells (Tregs) (2, 47); however, the role of parasite persistence in visceralizing forms of Leishmania infection is not clear. Although our data demonstrate that KBMA L. infantum chagasi parasites do not persist in mouse liver and spleen 6 months following intravenous injection, it is not known how long either live or KBMA organisms (or antigens) persist following subcutaneous inoculation. This subcutaneous vaccination with either live or KBMA promastigotes induced protection against visceral disease for at least 8 weeks following vaccination. As recent evidence suggests, the dosage and route of inoculation challenge may influence immunity significantly (33). Thus, further studies will be necessary to assess the longer-term durability of the protection.

The KBMA strategy merits comparison with the strategy of attenuating pathogens using ionizing radiation. Prophylactic immunization against experimental leishmaniasis using irradiated promastigotes dates back to the studies of Howard et al. and Rivier et al. (20, 37). One promising current candidate for a malarial vaccine is an irradiated, metabolically active, nonreplicating sporozoite vaccine, both of which provide the means to easily and broadly attenuate a population of pathogenic microbes. This strategy could ultimately be combined with more targeted attenuation strategies, such as deletion or modification of specific virulence genes, which by themselves result in strains prone to reversion in vivo. Additionally, novel vaccine adjuvants might also provide a means to boost effector and memory immune responses generated by attenuated whole-cell vaccines. It is likely that the development of clinically useful vaccines will ultimately combine multiple strategies to provide the necessary safety and efficacy for widespread human use. Ultimately, many questions regarding both the safety and efficacy of the KBMA approach must be addressed and investigated before KBMA vaccines could be considered for use in humans. However, we believe the data presented here support further research into this method as a viable approach to generate attenuated, immunogenic, and protective organisms for development into effective vaccine platforms.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants AI078431 (N.C.) and AI29646 (S.M.B.) as well as LA BioMed Seed funding (K.W.B.). We thank Peter Christenson for statistical assistance, Thomas Dubensky, Dirk Brockstedt, and John Hearst for provision of the S-59 and helpful discussions surrounding its use, and Jeff F. Miller at University of California Los Angeles for valuable guidance with early experiments and a robust discussion of results. We also thank Chaitra Marathe, David Piwnica-Worms, Julie Prior, and Kelly Robinson for technical assistance and helpful discussions.

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