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Killed but Metabolically Active *Leishmania infantum* as a Novel Whole-Cell Vaccine for Visceral Leishmaniasis

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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 intravenous 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-

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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/JL/80/Friedlin) clone V1, were engineered to express luciferase following integration of the construct pIR1SAT-LUC-TK (B5113) into the rRNA locus (45; also S. M. Hickerson and S. M. Beverley, 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% biotin, 0.25% hemin, 100 IU penicillin, 100 mg/ml streptomycin, 10% fetal bovine serum, and 0.25 mg/ml biopterin. The *L. infantum chagasi* strain MHOM/BR/00/1669 (previously known as *Leishmania chagasi*, and now considered synonymous with *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. Hemoflagellate 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/cm^2 UVA irradiation in an Intercept 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 coincubated 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 (10^7/100 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 10^7 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 FACSCalibur (BD) instrument.

Determination of organ 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 2×10^5, 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′-GTA CGG CTG CGA CAC CTT-3′; GP63 rev, 5′-AGC CGA GGT CCT GGA AGA T-3′; GP63 probe, 5′-/56-FAM-AGC CCG CAC CGC CCT GGT-36-TAMSp-3′ (where FAM is 6-carboxyfluorescein and TAMsp is 6-carboxytetramethylrhodamine); TNF-α for, 5′-TCC CTC TCA TCA GTT CTA TGG CCC A-3′; TNF-α rev, 5′-CAG CAA GCA TCT ATG CAC TTA GAC CC-3′; TNF-α probe, 5′-/56-JOEN-TGG AGG AAG GGC 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 (C_T) values below 35 were converted to absolute parasite counts using previously determined standard curves and were normalized to tissue DNA amounts by TNF C_T values, as previously described (9). GP63 C_T 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 3×10^6 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-phycoerythrin (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 manufactur-

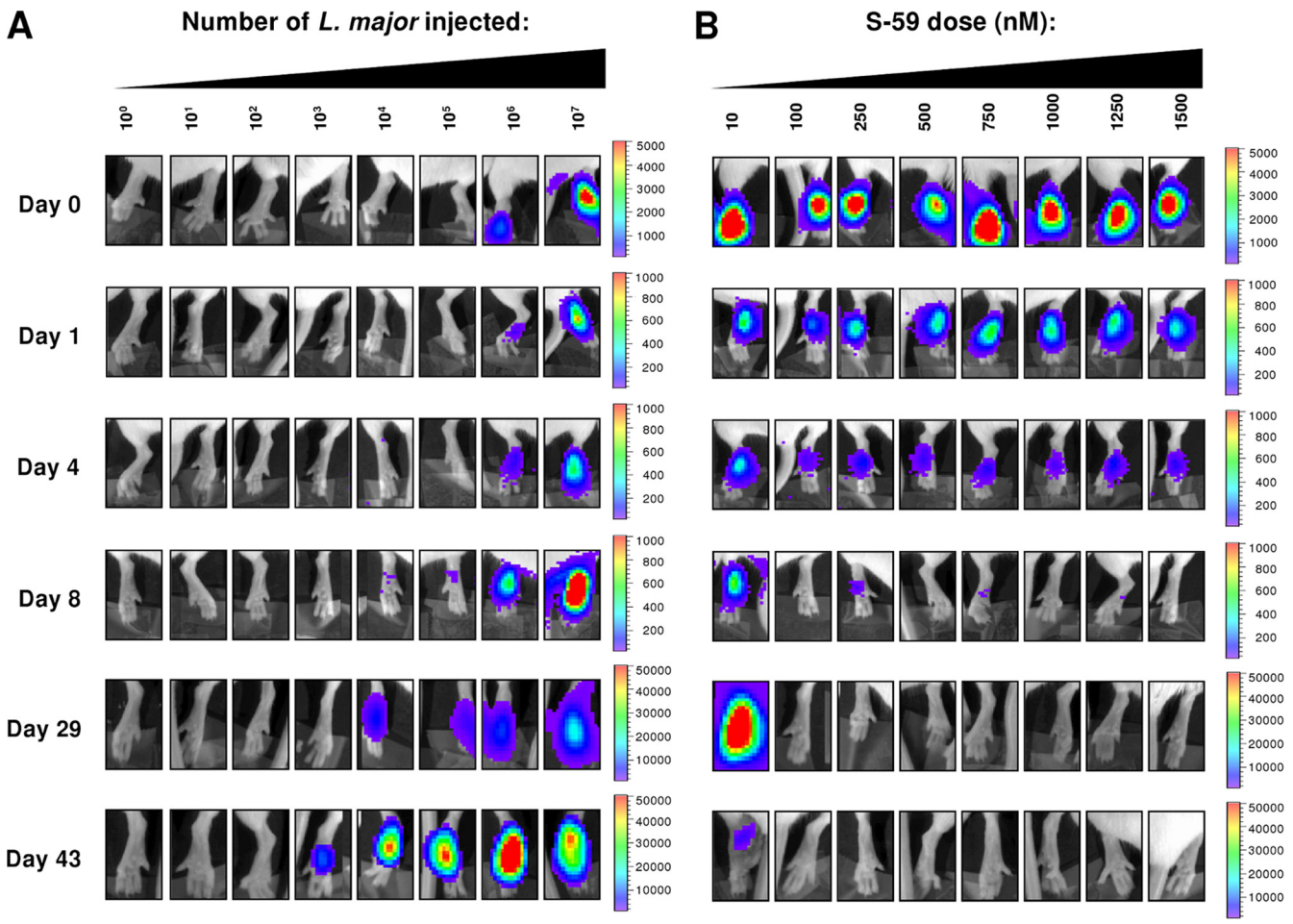

FIG 1 Treatment of *Leishmania major* promastigotes with S-59/UVA prevents progression of footpad infection. In the experiment shown in panel A, mice were injected with the indicated numbers of live, untreated luciferase-expressing *L. major* promastigotes to determine the sensitivity of the assay to detect outgrowth of live virulent parasites over time. Note that each individual footpad received a different inoculum of *L. major*. In the experiment shown in panel B, mice were injected with 10^7 luciferase-expressing *L. major* promastigotes that had been pretreated with increasing doses of S-59 (plus UVA). Note that each individual footpad was challenged with the same number of *L. major* parasites, but each inoculum of promastigotes had been treated with different doses of S-59.

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 of promastigotes (10^7) 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

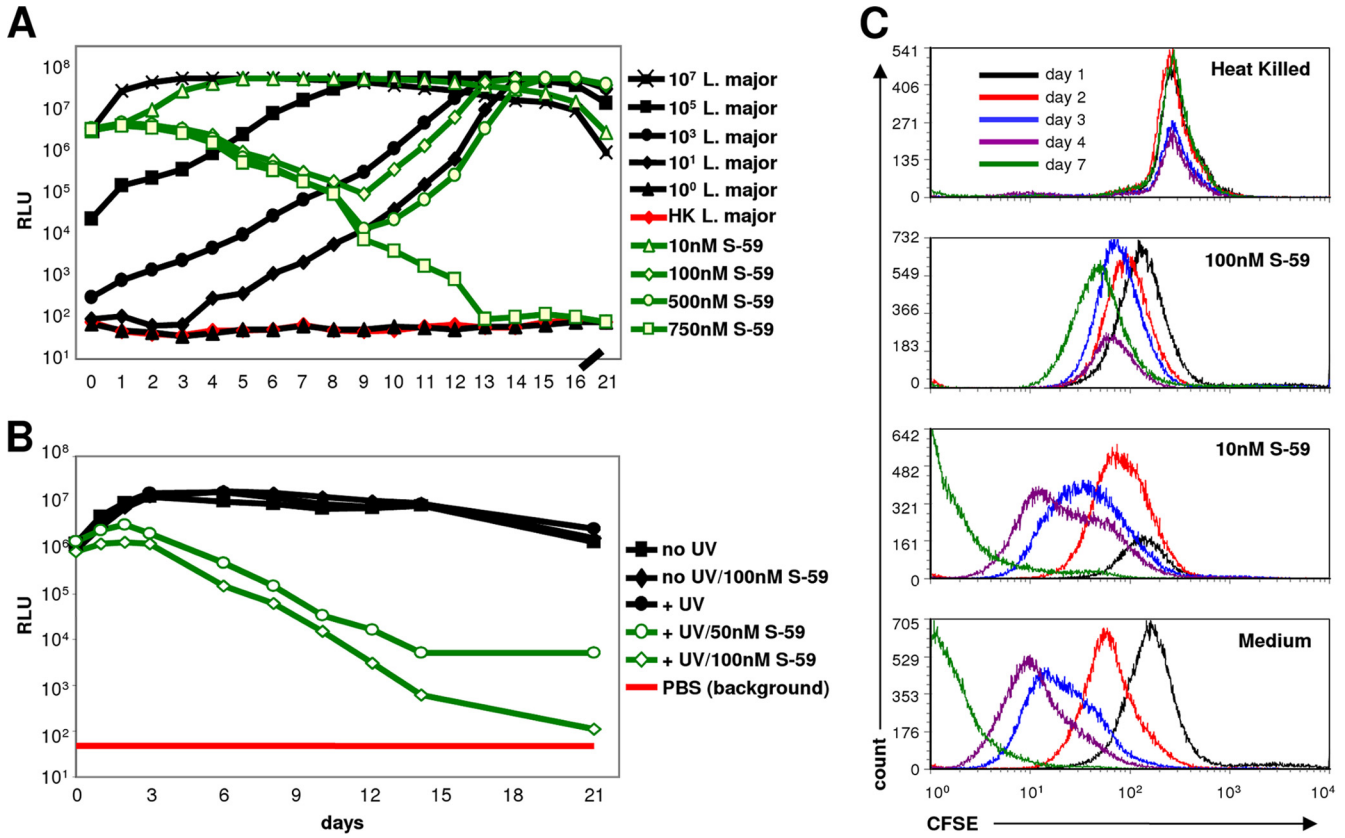

FIG 2 Treatment of different species of *Leishmania* with S-59/UVA leads to extinction of metabolic activity and replication arrest in vitro. (A) Indicated numbers of untreated *L. major*-luciferase promastigotes (black symbols) or 10^7 promastigotes treated with the indicated doses of S-59 (plus UVA) (green symbols) were cultured *in vitro* and sampled for luciferase activity at the time points shown. Luminescence of each lysed culture sample is reported in relative light units (RLU). (B) *L. infantum chagasi*-luciferase promastigotes were placed in culture at 10^7/ml following treatment with S-59 alone, UVA irradiation alone, or both, as shown. Luminescence from lysed samples of each culture was measured for the given time points. The threshold of detection (luminescence of PBS) is shown in red. (C) *L. infantum chagasi* promastigotes were stained with CFSE and treated with 10 nM or 100 nM S-59 (plus UVA) on day 0 and assessed by flow cytometry for CFSE levels at the time points shown. Controls were heat-killed promastigotes or untreated parasites (medium). HK, heat killed.

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 *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 ≥100 nM, combined with UVA irradiation, render *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 *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 *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 *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 *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 *L. infantum chagasi* yielded similar results. Cultures of *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 *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, *L. infantum chagasi* promastigotes were treated with 100 nM S-59 (plus UVA); they are referred to here as KBMA *L. infantum chagasi*.

KBMA *L. infantum chagasi* parasites do not persist or cause organomegaly *in vivo*. Virulent *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 *L. infantum chagasi* to persist long-term *in vivo*, we intravenously injected five BALB/c

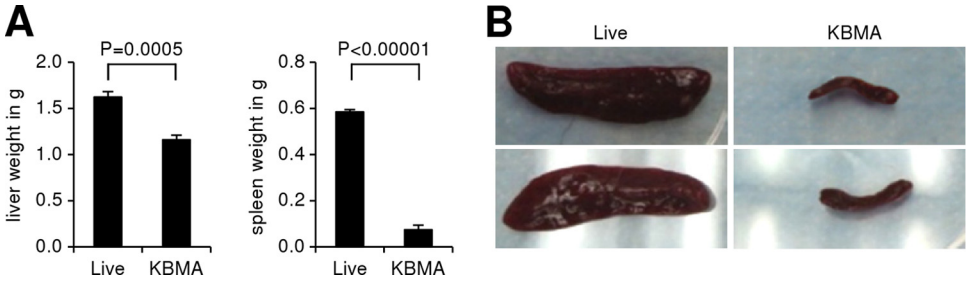

FIG 3 Primary infection with KBMA *L. infantum chagasi* does not result in long-term organomegaly in mice. Groups of five mice each were intravenously inoculated with either live or KBMA *L. infantum chagasi* promastigotes and 6 months later were euthanized. (A) Organs were removed and weighed. (B) Representative spleens from inoculated mice are shown. KBMA *L. infantum chagasi*-infected animals had spleens that appeared normal after 6 months.

mice each with 10^7 untreated live or KBMA *L. infantum chagasi* parasites. Six months after infection, all mice inoculated with live *L. infantum chagasi* displayed marked hepatosplenomegaly, while all of the KBMA *L. infantum 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 *L. infantum chagasi*-infected mice. No parasites, however, could be visualized microscopically in any of the mice infected with KBMA *L. infantum chagasi*. Quantitative PCR is a more sensitive method of detection and demonstrated large parasite loads in the livers and spleens of live *L. infantum chagasi*-infected animals. However, we were unable to detect any *Leishmania* DNA in the organs of KBMA *L. infantum chagasi*-infected animals (data not shown). Thus, while live *L. infantum chagasi* infection caused organomegaly and parasite persistence in mouse liver and spleen 6 months after infection, KBMA *L. infantum chagasi* parasites were undetectable in tissues at this time point, and KBMA *L. infantum chagasi*-infected animals showed no signs of associated organomegaly or detectable parasite presence.

KBMA *L. infantum chagasi* parasites enter macrophages and induce NO production. *Leishmania* are capable of infecting macrophages both *in vivo* and *in vitro*. Entry of parasites into macrophages leads to induction of iNOS expression and secretion of NO in macrophages primed with IFN-γ (17). To determine if KBMA *L. infantum chagasi* parasites also induce a similar macrophage response, we exposed primary murine bone marrow-derived macrophages (BMDM) from BALB/c mice to *Leishmania in vitro* and measured supernatant levels of NO. In the presence of exogenously added IFN-γ, KBMA *L. infantum chagasi* induced NO production at levels similar to live *L. infantum chagasi* (Fig. 4A). We performed similar infections of BMDM in the absence of exogenous IFN-γ to allow for the persistence of intracellular amastigotes. Both live promastigotes and KBMA *L. infantum chagasi* entered BMDM *in vitro*, transformed into amastigotes that were

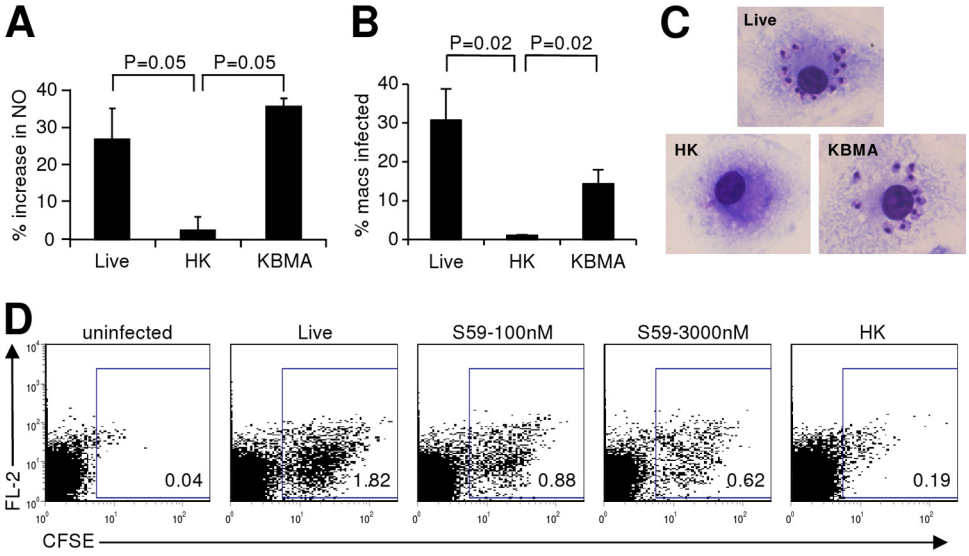

FIG 4 KBMA *L. infantum chagasi* parasites enter cells and induce NO. (A) BMDM were infected with *L. infantum chagasi* promastigotes at an MOI of 20 in the presence of 10 U/ml IFN-γ. Levels of NO production at 48 h are shown as a percent increase compared to IFN-γ stimulation alone. Bars represent the means of three independent experiments, with standard errors of the means. (B) BMDM were infected with *L. infantum chagasi* promastigotes at an MOI of 20, with no exogenous IFN-γ added. The percentage of infected macrophages was counted after 48 h. Bars represent means ± standard errors of the means of triplicate infections from a representative experiment. (C) Examples of infected Giemsa-stained cells from panel B. (D) CFSE-labeled *L. infantum chagasi* parasites were treated with doses of 100 nM and 3,000 nM S-59 (plus UVA) or heat killed (HK) and injected intravenously into BALB/c mice. Twenty-four hours later livers were harvested, and single-cell suspensions were analyzed by flow cytometry for CFSE positivity. Free parasites were excluded using forward scatter gating. Percentages of cells containing CFSE-positive organisms are indicated within the gates. Results are representative of two independent experiments.

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 KMBA *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 10^7 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*.

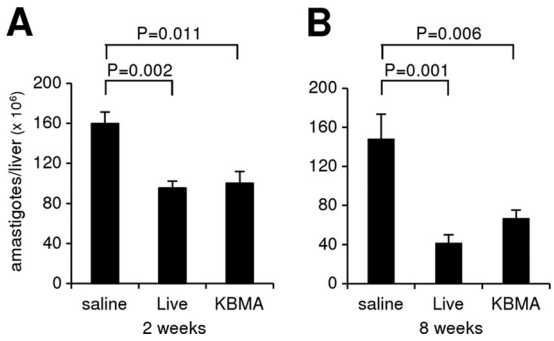

FIG 5 Subcutaneous vaccination with KBMA *L. infantum chagasi* confers protection to mice against challenge with virulent *Leishmania*. Groups of eight mice each were vaccinated and boosted subcutaneously a total of three times, every 2 weeks, with either live or KBMA *L. infantum chagasi* and then challenged intravenously with live, virulent *L. infantum chagasi* 2 weeks (A) or 8 weeks (B) later. Liver parasite burdens were measured 28 days following challenge by microscopic quantitation of Giemsa-stained liver preparations. Control mice that were vaccinated but not challenged intravenously with virulent parasites had no detectable parasites by microscopy in the touch preparations after 1 month (data not shown). Data are means ± standard errors of the means and are representative of two independent experiments.

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

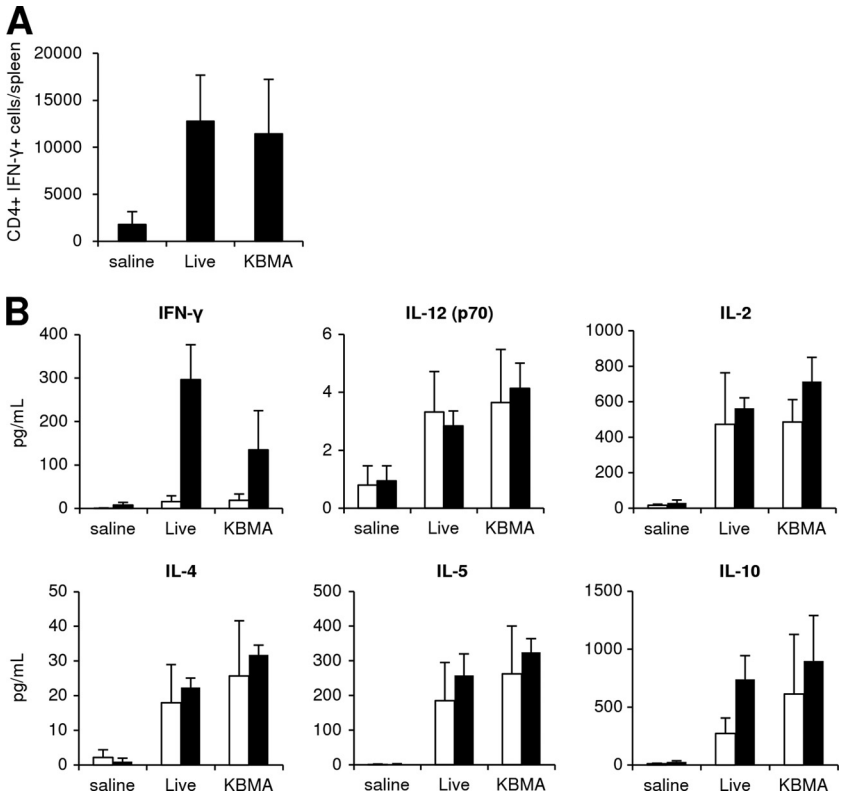

FIG 6 Vaccination with KBMA *L. infantum chagasi* promastigotes induces *Leishmania*-specific splenic T cell responses. (A) Splenocytes from mice subcutaneously vaccinated with live or KBMA *L. infantum chagasi* parasites (as described in the legend of Fig. 5) were harvested 2 weeks after the final vaccination. Single-cell spleen suspensions were stimulated *in vitro* with live *Leishmania* parasites for 3 days in the presence of brefeldin A and assessed by flow cytometry for intracellular IFN-γ production. Data are the means ± standard errors of the means ($n = 3$ mice per group). (B) Splenocytes from mice vaccinated with live or KBMA *L. infantum chagasi* were harvested 2 weeks after the final vaccination and cultured for 3 days in the presence of live *L. infantum chagasi* promastigotes (black bars) or medium only (white bars). Supernatants from these cultures were analyzed for cytokine concentrations using a Luminex multiplex cytometric bead assay. Bars show concentration of the relevant cytokine in pg/ml ± standard errors of the means ($n = 3$ mice per group).

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 reinfection 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 antigen) 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 *P. falciparum* sporozoite (PfSPZ), under development by Sanaria (13). Biochemically, a main distinction between the two approaches is that the psoralen-based strategy generates relatively few permanent double-stranded DNA cross-links that prevent replication by physically preventing strand separation, while ionizing radiation strategies induce broad DNA damage that inhibits viability. It remains to be seen whether this biochemical difference will affect the ultimate immunogenicity of either vaccine method. Our data demonstrate that KBMA-attenuated *Leishmania* strains develop into the amastigote form inside macrophages. This finding is similar to radiation-attenuated *L. major* promastigotes (1, 21, 37) and the *P. falciparum* sporozoite vaccine, both of which demonstrate the ability to enter a target cell and undergo an initial life cycle transformation. This process is likely important for overall immunogenicity.

The fact that some asymptomatic patients develop a protective immune response after natural infection with *Leishmania* species supports the case for development of safe, whole-cell vaccines. Diverse host immune responses and the capacity of protozoa to modify or vary their surface antigens suggest that a diverse antigenic repertoire in a vaccine may be required for widespread public use. Given that over half the vaccines approved by the U.S. Food and Drug Administration follow a live, attenuated, whole-cell strategy (19), expanding the technological methods for this general approach is worthwhile. The KBMA-based strategy provides 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.

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