Production of Live Attenuated *Leishmania major* Cell Banks under Current Good Manufacturing Practices (cGMP) for Vaccine Development against Leishmaniasis.

AFS ATCC FEDERAL SOLUTIONS

MHSRS-23-08788

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BACKGROUND

Leishmaniasis is a vector-borne neglected tropical disease caused by protozoan parasites of the genus Leishmania. The disease is transmitted by parasite-infected sand flies and is endemic to regions of Africa, Asia, Southern Europe, and Latin America. In the United States, leishmaniasis has been historically associated with military deployment, especially to Iraq, with the largest number of American cases recorded between 2002 and 2016. The exposure risk to Leishmania infection among the U.S. armed forces remains significant due to the possibility of future deployments to the Middle East and other endemic regions. Current therapies exhibit toxic effects and may lead to drug resistance; therefore, a vaccine would be the most optimal approach to prevent disease.

A concerted effort by researchers from academia and the FDA generated a centrin gene-deleted live attenuated *Leishmania major* dermotropic parasite (*LmCen*$^{-/-}$). The parasite is safe and protective in animal models and is compatible with human trials as a live attenuated vaccine. Importantly, future clinical studies of the vaccine candidate will require production under current good manufacturing practices (cGMP). The present work describes completion of the first of these steps, namely the production and characterization of master cell banks (MCB) and working cell banks (WCB) of *LmCen*$^{-/-}$ parasites according to cGMP guidelines. The processes put in place will serve as the framework for manufacturing the standardized cGMP-grade live attenuated vaccine to be tested in human studies.

TECHNICAL APPROACH

Characterization of Research Cell Banks (RCB). RCBs delivered to the ATCC from Gennova Biopharmaceuticals underwent pre-bank testing prior to the production of MCB (Fig. 1). Tests included growth and viability studies, cell morphology analysis, sterility tests to rule out bacterial or fungal contamination, PCR-based tests for mycoplasma and >30 bacterial and viral human pathogens, evidence of *L. major* centrin deletion by PCR, and confirmation of the parasite species by DNA sequencing of the *Leishmania* ITS1/ITS2 regions and the *nagt* gene.

Fig. 1. Pre-bank Testing of *LmCen*$^{-/-}$ RBC

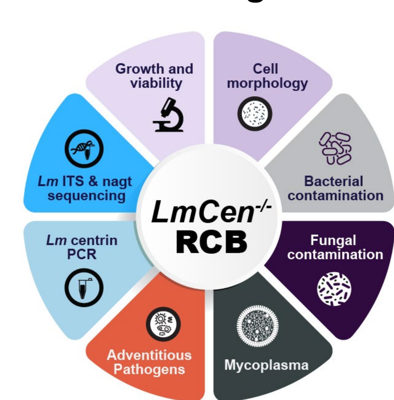

Fig. 1. *LmCen*$^{-/-}$ RCB was subjected to a panel of pre-banking tests to verify the identity of the line and rule out the presence of contaminating agents prior to manufacturing of the MCB.

Production of and Characterization of MCB and WCB. cGMP-grade *LmCen*$^{-/-}$ MCB was produced in RPMI-1640 medium supplemented with panhematin, adenosine, folic acid, biotin, and 10% FBS. Seed cultivation was initiated from pre-bank tested *LmCen*$^{-/-}$ RCB in T25 cm^2 culture flasks for 3-4 days at 27°C and expanded to T75 cm^2 flasks. After 3-4 days of growth, cultures were expanded to ~300 mL of medium and incubated until stationary phase and a maximum cell density of 3-5 x 10^7 cells/mL. Parasites were harvested by centrifugation, and MCB vials were filled at a density of 1 x 10^7 cells/mL. The *LmCen*$^{-/-}$ MCB underwent the same characterization tests as the RCB with the addition of assays listed in Table 1. *LmCen*$^{-/-}$ WCB was produced from post-bank quality controlled (QC) MCB. Growth and viability studies of the WCB, sterility tests in selective media, macrophage and metacyclic assays, whole genome sequencing (WGS), and other molecular tests were performed similarly to the MCB. All laboratory procedures, documentation practices, and cryostorage conditions used in cell banking were performed according to guidelines found in FDA 21CFR1271 - Human Cells, Tissues, and Cellular and Tissue-Based Products.

Table 1. Characterization Testing of cGMP grade *LmCen*$^{-/-}$ Cell Banks

Test	Specifications	Supporting Data
Cell Density and Viability (Post-cryopreservation)		
Cell count	10^7 cells/mL	Cell density determined microscopically upon thawing.
Culture Density and Viability	Growth of Leishmania promastigotes in RPMI. Cell density and viability determined microscopically at 0, 24, 48, 72, 96, 120 h after inoculation.	Cultures reach ~3-5 x 10^7 cells/mL after 5 days. Viability ≥85% by Trypan Blue dye exclusion analysis.
Identity and Stability Tests		
Cellular morphology	Microscopic observation of promastigotes in culture	Presence of lance-shaped promastigotes and small rosettes in culture, consistent with genus.
Whole Genome Sequencing	≥99% match with *Leishmania major* Friedlin V1; deletion of centrin gene	Identical genetic makeup with *LmCen*$^{-/-}$ RCB
Phenotypic tests		
Infectivity *in vitro*	Growth in THP-1 macrophage cell line (ATCC® TIB-202™) determined at 6, 48, and decrease by 120 h p.i.	Microscopic analysis show a decrease in the percentage of infected macrophages by 120 h p.i. and multinucleated intracellular amastigotes.
Formation of metacyclics	Estimation of metacyclic population in culture by peanut lectin agglutination (PNA) test	Microscopic counts show ~20-30% metacyclics as determined after 3-5 days of culture.

Table 1. Characterization Testing of cGMP grade *LmCen*$^{-/-}$ Cell Banks (Continued)

Test	Specifications	Supporting Data
Tests for Purity and Contamination		
Sterility Media Panel (21 day): 7 microbial culture media, two temperatures (26 °C and 37 °C), two Atmospheric conditions (aerobic and anaerobic)	No growth of bacteria or fungi	No growth of microbial contaminants in 7 culture media after 7, 14, and 21 days of incubation. Sterility validation by bacteriostasis and fungistasis testing.
Mycoplasma screening - Direct culture	Negative	No growth on *Mycoplasma* agar, broth
Mycoplasma screening - PCR – ATCC® UMDK2	Negative	No PCR product detected
Endotoxin test - Kinetic Chromogenic LAL Assay	Negative	<0.500 EU/ml
Adventitious pathogen screening – Testing of a panel of 60 human, hamster, and mouse viral and bacterial pathogens by qPCR	Negative	No detection of 60 pathogens by qPCR
Leishmania RNA Virus (LRV) testing by RT-PCR	Negative	No detection of LRV2

CELL BANK CHARACTERIZATION TESTS

Fig. 2. Cell Density and Viability

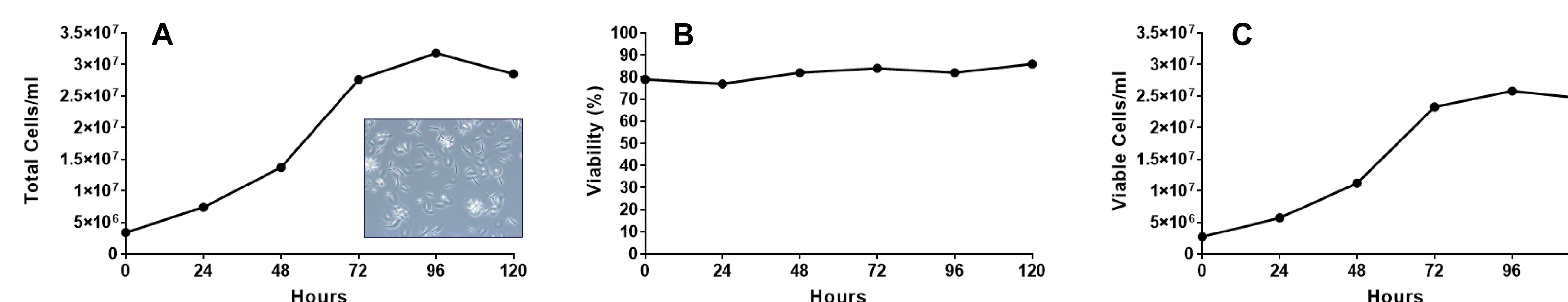

Fig. 2. Assessment of Cell Density and Viability of cGMP-grade *LmCen*$^{-/-}$ Cell Banks. A cryostock of *LmCen*$^{-/-}$ MCB (1 x 10^7 cells/mL) was thawed and cultured in a T25 cm^2 flask with 5 mL RPMI medium supplemented with panhematin, adenosine, folic acid, biotin, and 10% FBS. Cultures were incubated at 27°C and microscopic cell counts were performed every 24 h using a hemocytometer **(A).** Inset shows a representative image of promastigotes cultured for 72 h. Cell viability was determined by Trypan blue dye exclusion analysis **(B, C).** Values for each time point represent the averages of cell counts performed in duplicate.

Fig. 3. PCR and RT-PCR-based testing of *LmCen*$^{-/-}$ Cell Banks

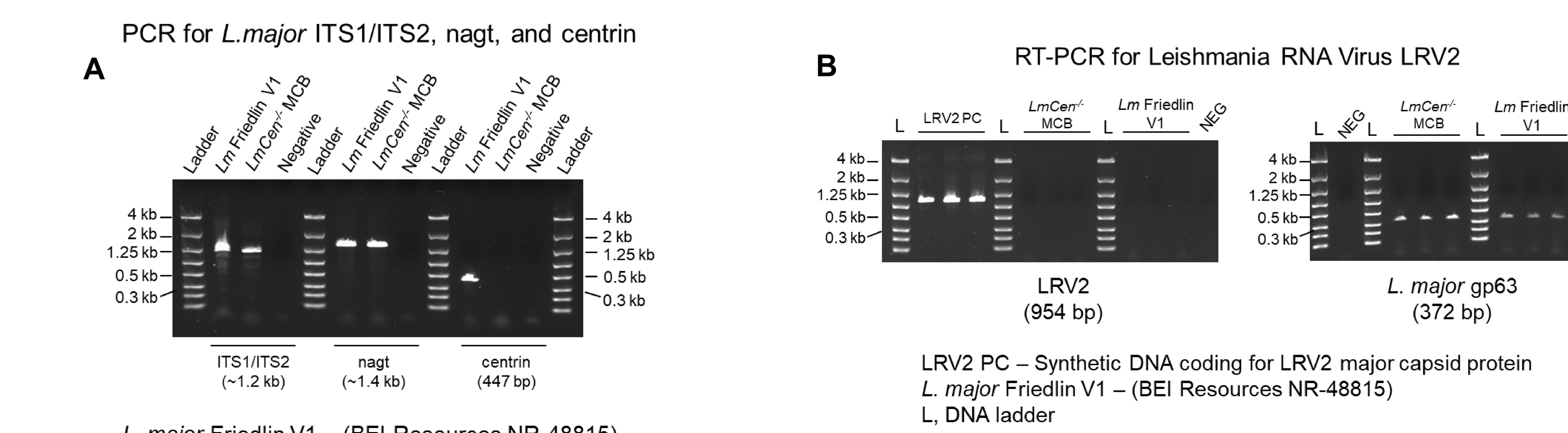

Fig. 3. PCR and RT-PCR-based testing of cGMP-grade *LmCen*$^{-/-}$ MCB. PCR of the *Leishmania* ITS1/ITS2 regions and nagt gene followed by DNA sequencing of the amplicons was performed to confirm the species of the MCB as *L. major* **(A)**. The deletion of the centrin gene was also confirmed by PCR in the attenuated *LmCen*$^{-/-}$ line as compared to the wild type *L. major* Friedlin V1 parental strain (BEI Resources NR-48815). RT-PCR was performed in *LmCen*$^{-/-}$ MCB to rule out the presence of the *Leishmania* RNA virus LRV2 **(B)**. Both the attenuated *LmCen*$^{-/-}$ line and the wild type *L. major* Friedlin V1 were negative, as expected. The expression of *L. major* gp63 was used as a control for RT-PCR.

Fig. 4. Whole Genome Sequencing of cGMP-grade *LmCen*$^{-/-}$ MCB

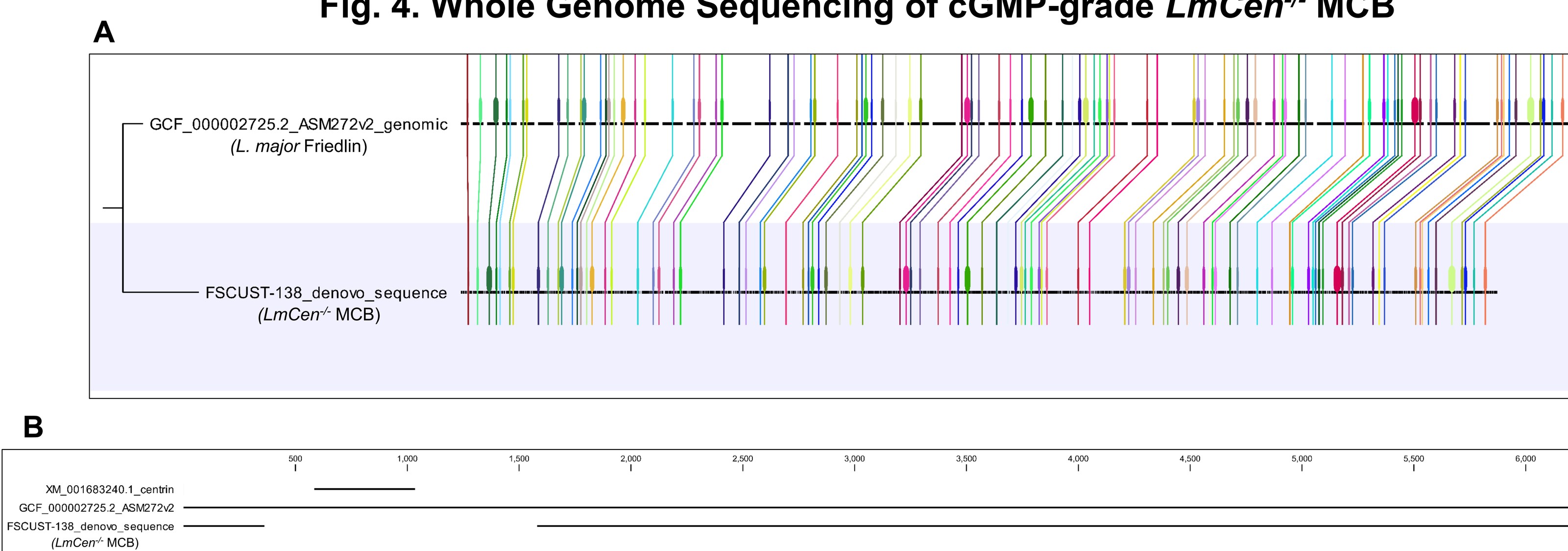

Fig. 4. Whole Genome Sequencing of cGMP-grade *LmCen*$^{-/-}$ MCB. A, Whole genome alignment of *Leishmania major* strain Friedlin reference sequence (GCF_000002725.2) and the *LmCen*$^{-/-}$ MCB de novo sequence (FSCUST-138). The lines running vertically indicate areas of high similarity between the two sequences. **B,** Sequence alignment of the centrin gene (XM_001683240.1), the *Leishmania major* strain Friedlin reference sequence (GCF_000002725.2), and the *LmCen*$^{-/-}$ MCB de novo sequence (FSCUST-138). Based on the alignment, the centrin gene is only present in the Friedlin reference sequence but absent in *LmCen*$^{-/-}$ MCB .

CELL BANK CHARACTERIZATION TESTS

Fig. 5. *In vitro* Infection of cGMP-grade *LmCen*$^{-/-}$ Cell Banks in Macrophages

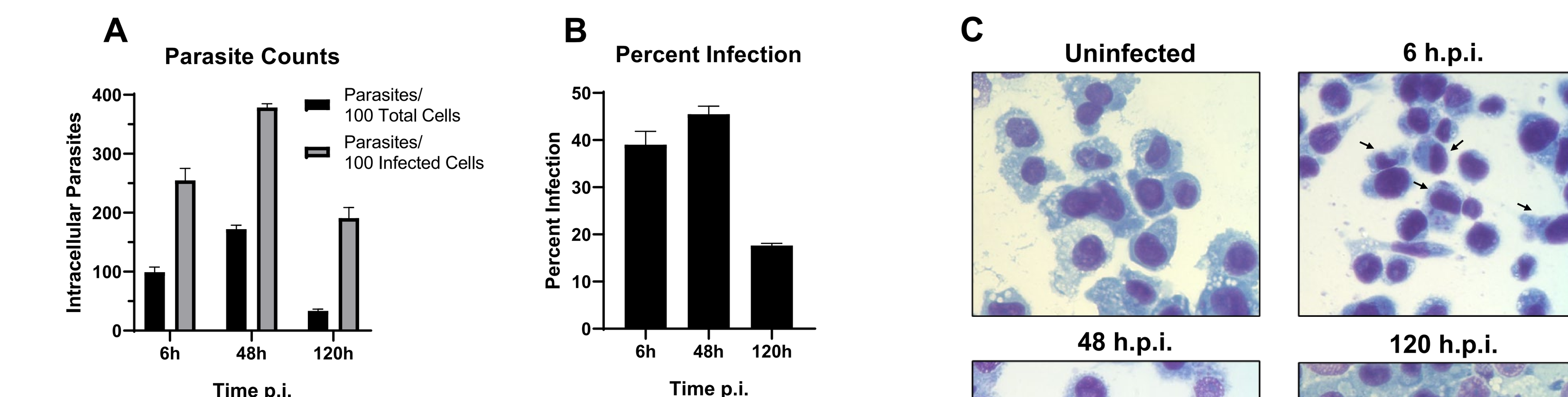

Fig. 5. Assessment of macrophage infectivity by *LmCen*$^{-/-}$ WCB. A cryostock of *LmCen*$^{-/-}$ WCB was thawed and inoculated on THP1-derived macrophages. Uptake of promastigotes was allowed for for 6 h at 32°C/5% CO_2. Macrophages were subsequently incubated at 37°C/5%CO_2 for 6, 48, and 120 h. Quantification of intracellular amastigotes **(A)** and percentages of infected cels **(B)** were performed in Giemsa-stained macrophages under light microscopy. Data in **A** and **B** represent the means±SD of cell counts performed in triplicate. Panel **C** shows representative images intracellular amastigotes in macrophages (arrows).

Fig. 6. Estimation of Metacyclic Populations in cGMP-grade *LmCen*$^{-/-}$ MCB and *LmCen*$^{-/-}$ WCB

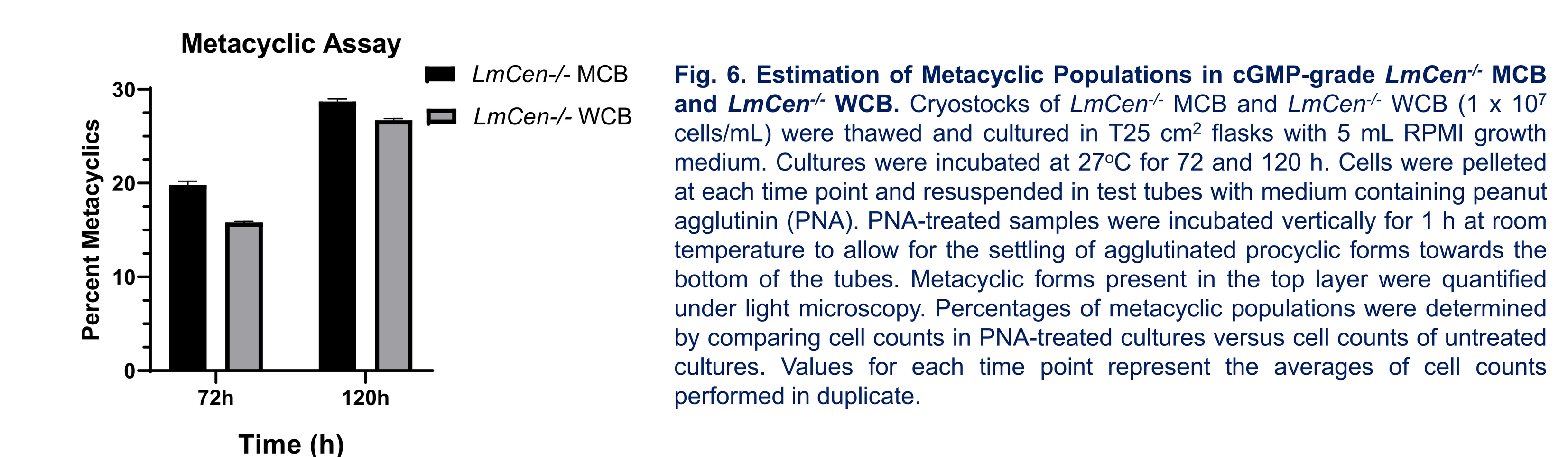

Fig. 6. Estimation of Metacyclic Populations in cGMP-grade *LmCen*$^{-/-}$ MCB and *LmCen*$^{-/-}$ WCB. Cryostocks of *LmCen*$^{-/-}$ MCB and *LmCen*$^{-/-}$ WCB (1 x 10^7 cells/mL) were thawed and cultured in T25 cm^2 flasks with 5 mL RPMI growth medium. Cultures were incubated at 27°C for 72 and 120 h. Cells were pelleted at each time point and resuspended in test tubes with medium containing peanut agglutinin (PNA). PNA-treated samples were incubated vertically for 1 h at room temperature to allow for the settling of agglutinated procyclic forms towards the bottom of the tubes. Metacyclic forms present in the top layer were quantified under light microscopy. Percentages of metacyclic populations were determined by comparing cell counts in PNA-treated cultures versus cell counts of untreated cultures. Values for each time point represent the averages of cell counts performed in duplicate.

SUMMARY

- *LmCen*$^{-/-}$ parasites cultured from MCB and WCB showed similar *in vitro* growth curve patterns, with characteristic progression through logarithmic and stationary growth phases.
- MCB and WCB were free of microbial contamination by validated sterility assays as required by all major pharmacopoeias.
- WGS analysis confirmed the genetic identity of the cGMP-grade *LmCen*$^{-/-}$ MCB and WCB to the previously published *L. major* Friedlin strain and *LmCen*$^{-/-}$ RCB.
- Macrophage infectivity assays of cGMP banks of *LmCen*$^{-/-}$ showed decreases in the percentages of infected cells and intracellular amastigotes over time. Amastigotes showed large, multi-nucleated cells indicative of deficient cell division, as expected.
- Molecular testing for adventitious agents confirmed that the MCB and WCB were free of human, porcine, bovine, and murine adventitious viral pathogens and LRV2.
- This work demonstrates that manufacturing *LmCen*$^{-/-}$ parasites on a large scale is feasible under cGMP. MCB and WCB passed all quality-controlled release tests, making this endeavor the preliminary stage to develop the platform for producing a live attenuated parasite line to be tested as a vaccine candidate against leishmaniasis.

ACKNOWLEDGEMENTS

This work was performed with funds from the Global Health Innovative Technology Fund (GHIT) Japan, Gennova Biopharmaceuticals, and the ATCC Internal Research and Development Program.

The following strain was obtained from the BEI Resources Repository, NIAID, NIH (www.beiresources.org): *Leishmania major* NIH Friedlin V1 (MHOM/IL/80/FN) (BEI NR-48815).

We thank the ATCC Sequencing & Bioinformatics Center for the WGS analysis of the *LmCen*$^{-/-}$ cell banks.

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