An Effective Live Vaccine Strain Of Trypanosoma Cruzi Prevents Chagas Disease In The Mouse Model

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Abstract

*Trypanosoma cruzi* is the etiologic agent of Chagas disease for which there are no prophylactic vaccines. Cyclophilin 19 is a secreted cis-trans peptidyl isomerase expressed in all life stages of *Trypanosoma cruzi*, which in the insect stage leads to the inactivation of insect anti-parasitic peptides and parasite transformation and in intracellular amastigotes participates in generating ROS enhancing parasite growth. We have generated a parasite knock-out mutant of Cyp19 which fails to replicate in cell culture or in mice indicating that lack of Cyp19 is critical for infectivity. Knock-out parasites fail to replicate in or cause clinical disease in immune-deficient mice further validating their lack of virulence. Repeated inoculation of knock-out parasites into immuno-competent mice elicits parasite-specific antibodies and T-cell responses. Challenge of immunized mice with wild-type parasites is 100% effective at preventing disease. These results indicate that the knock-out parasite line is a live vaccine candidate for Chagas disease.

Introduction

The protozoan parasite *Trypanosoma cruzi* is the etiologic agent of Chagas disease, which is endemic in Mexico, Central and South America. People living in regions within the lower United States, particularly in Arizona, New Mexico and Texas are also at increased risk for acquiring the disease. The disease is transmitted in multiple ways, chiefly by the feeding of hematophagous triatomine insects on mammalian hosts. Humans are an incidental hosts and those living rural areas are at greatest risk where poor housing conditions lead to the entrance and feeding of triatomines into houses. Feeding leads to the deposition of fecal material containing infective metacyclic-trypomastigote parasitic forms that end the contamination of mucosae around the feeding sites. Metacyclics bind to and invade host cells wherein they transform into and multiply in the host cell cytosol as intracellular amastigotes. Amastigotes transform thereafter into motile trypomastigotes that exit host cells, which circulate through the body and further infect cells in a variety of target organs. Chronic infection of organs, particularly cardiac myocytes and gastrointestinal smooth muscle, cause the sequelae of chronic Chagas disease. Additional modes of infection are by oral ingestion of parasite infected items, trans-placental maternal-fetal transmission and through transplant of infected blood and tissues to non-infected recipients. Importantly, the global transmigration of chronically infected hosts leads to the diagnosis of Chagas disease in countries that are not endemic for Chagas. Approximately 20–30% of chronically infected individuals develop chronic symptoms, which include Chagas-associated cardiomyopathy, due to chronic inflammation of cardiac tissue due to direct parasite damage and molecular mimicry. Chagas disease is the leading cause of heart failure in Latin America. Mega-organ syndromes of the esophagus and large intestine arise from denervation of the gastrointestinal tract due to direct or indirect damage of the smooth muscle.

Upon initial infection, there is limited exposure of the immune system to parasite antigens, since the inoculum is small and rapidly becomes intracellular. As parasites replicate intracellularly and then exit
host cells in larger numbers immune activation occurs. The immune response to *T. cruzi* is complex, but involves the induction of both Th1 and Th2 responses. Th-1 responses are signaled by the production of IL-12 leading to IFN-γ and TNF-α and NO production by macrophages. Activated inflammatory cells produce IL-1, -6 and −18 and are poised to kill intracellular parasites. Anti-inflammatory Th2 responses are also activated in order to control over-aggressive immune activation and involve IL-4 and −10 production. Additionally CD8+ lymphocytes and anti-parasite IgG is also produced which limit the increase in parasite load. Chronic smoldering infection ensues after several years due to several factors eventually leading to chronic sequelae of Chagas disease in about one-third of infected patients. It is unclear why only a subset of infected individuals develops these chronic manifestations, but this is likely due to complex interplay host genetic and parasite factors. Treatment of Chagas disease is limited to two drugs, nifurtimox and benznidazole, with considerable side effects. There are no vaccines available for prevention of Chagas disease.

The virulence of *T. cruzi* is dependent on a myriad of protein-, lipid- and glycan-based virulence factors, which lead to the binding, invasion and intracellular replication of parasites in both phagocytic and non-phagocytic cells. A number of years ago our laboratory identified a secreted cis-trans peptidyl-prolyl isomerase, termed cyclophilin 19, which was expressed by insect stage *T. cruzi* and promoted the resistance of parasites to killing by insect anti-microbial peptides as well as induction of parasite calcineurin leading to partial transformation of parasites into metacyclic forms and enhancement of infectivity. Since then we have found that cyclophilin 19 is expressed by and secreted by all parasite stages including intracellular amastigotes, where is promotes the reactive oxygen species important for intracellular parasite growth and survival (Pedroso, et al, unpublished). Cyclophilins are a highly conserved family of enzymes, which play a myriad of roles in biology, mainly acting as protein chaperones by isomerization of proline residues within protein substrates. Aberrant expression of cyclophilins, particularly the closest human homologue to cyclophilin 19, cyclophilin A, is associated with enhanced invasiveness of certain malignant tumors and the induction of cardiac inflammation. The binding and isomerization of proline within the cell signaling ligand, CD147 by extracellular cyclophilin A, leading to its activation, is mechanistically implicated in these phenotypes.

The genome sequence of *T. cruzi* indicated that cyclophilin 19 is encoded by a single-copy gene, so in order to determine the role of cyclophilin 19 in parasite virulence and pathogenesis, we employed a double-allelic homologous recombination gene knock-out strategy to remove cyclophilin 19 genes from parasites. Double knock-out parasite mutants with depleted cyclophilin 19 were unable to infect host cells in vitro or mice in vivo. Repeated infection of mice with these mutants led to increasing levels of parasite specific T- and B-cell responses that were 100% effective at preventing death of acute Chagas disease in mice indicating this may be an effective vaccine strain for prevention of Chagas disease. The mutant strain did not replicate in immune-deficient mice strains and dexamethasone treatment of mice infected with the mutant strain did not develop clinical disease or the emergence of parasites, indicating that the vaccine strain is safe in immune-suppressed host conditions. Lastly, a single dose of this attenuated parasite line led to complete protection of high dose challenge with highly virulent wild type parasites.
Overall, this data is proof of principle that this mutant line is an effective and safe live vaccine strain against Chagas disease.

**Materials And Methods**

**Parasites and cells.** *T. cruzi* Brazil strain epimastigotes were cultured at 26°C in liver digest-neutralized tryptose (LDNT) medium supplemented with 10% fetal bovine serum (FBS), 20 µg/ml hemin, 100 µg/ml streptomycin, and 100 U/ml penicillin. Early to mid-log-phase parasites were used for the experiments. Transformed clones of Neomycin and Hygromycin resistant cells were maintained at a concentration of 200 µg/ml of G418 and Hygromycin B in LDNT medium. Rat Heart Myoblast (H9C2) cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin at 37°C in a humidified atmosphere containing 5% CO₂. Tissue culture trypomastigotes were recovered from the supernatant of infected monolayers of H9C2 cells.

**Animals.** All animal experimental protocols were approved by the Ethical Committee for Animal Research of the Ohio State University. Mice were maintained under a 12-hour light-dark cycle. The animals were maintained according to the rules and regulations of The University Laboratory Animal Resources (ULAR). *T. cruzi* Brazil strain parasites were maintained in AJ mice (Jackson Laboratories, Bar Harbor, ME, USA). Male AJ mice were infected with 1 · 10⁵ trypomastigotes, unless otherwise stated, for all the experiments.

**Plasmid constructs preparation.** Plasmid pET15b was used to delete TcCyp19 using Ndel and BamHl restriction sites. Homologous nucleotide sequences of 5’UTR and 3’UTR were generated by PCR using genomic DNA template. The PCR primers used to amplify 442 bp of 5’UTR were (forward, Ndel) 5’-GTCTACTACTACCATCCCAAGG-3’ and (reverse, Xhol) 5’-TGTGTTGTTAATAAATTTTTC-3’, and PCR primers used to amplify 177 bp of 3’UTR were (forward, Sall) 5’-ACTGCTCTTCCGCGAGCTTTTG-3’ and (reverse, BamHl) 5’-GTATAAGTCAGTTTTACACCC-3’. PCR primer pairs (forward, Xhol) 5’-ATGATTGAACAGATGGATTGC-3’ and (reverse, Sall) 5’-TCAGAAGACTCGTCAAGAAGG-3’, were used to amplify 795 bp of neomycin phosphotransferase (Neo), and PCR primer pairs (forward, Xhol) 5’-ATGAAAAGCCTGAACCTACCC-3’ and (reverse, Sall) 5’-CTATTTCCTTGGCCCTCGGACG-3’ were used to amplify 1026 bp of hygromycin phosphotransferase (Hyg). The amplified products of 5’UTR, 3’UTR, Neo and Hyg were cloned into pGEM®-T Easy (Promega, Madison, WI, USA) vector. The gene cassettes of 5’UTR + Neo/Hyg + 3’UTR were constructed in pET15b plasmid that was extracted and purified using plasmid maxi prep kit columns (Qiagen).

**Genomic DNA Isolation.** Epimastigotes from *T. cruzi* wild type (WT) and cyp19 knock out (KO) were collected from the culture and washed twice with PBS. Genomic total DNA was extracted using DNeasy Blood & Tissue Kits (Qiagen) according to the described manufacturer’s protocol. PCR was performed to amplify various targets to clone into plasmids and to confirm the presence and absence of genes.
Transfection, selection and cloning of *T. cruzi*. A total of $1 \times 10^7$ early log phase of *T. cruzi* epimastigotes was used to transfec with 50 µg of linearized Neo/Hyg plasmid using the electroporator (Harvard Apparatus BTX, Holliston, MA). Transfected parasites were maintained for 24 h in LDNT medium alone. The selection pressure was created with 200 µg/ml of G418 and 200 µg/ml of Hygromycin B for transfectants with neomycin phosphotransferase and hygromycin phosphotransferase gene cassette, respectively. Parasites were selected for 4–5 weeks post-transfection and considered fully selected when all the parasites with no resistant marker gene cassette were dead. Individual clones were obtained by limiting dilution into a 96-well plate. Individual cloned cells were further analyzed for the absence of cyp19 gene.

Nuclear genomic sequencing and analysis. The libraries were sequenced using a PE75 protocol (75-bp reads from each paired end) on a HiSeq2000 at the UW Sequencing Northwest Genomics Center. After de-indexing, we obtained between 21.2M (JM255) and 44.1M (JM256) reads per library. Aligned all reads from the 7 WGS libraries against the 43 chromosomes of the TcBrA4 genome from TriTrypDBv46 using Geneious assembler containing the following loci: 1) Cyp19: A 21,328-bp fragment from PRFA01000019 (reverse complemented) containing three genes on either side of C4B63_19g183), which is the orthologue of TcCLB.506925.300. This locus is on Chr39-S in CL Brener; 2) Cyp11: A 12,966-bp fragment from PRFA01000149 containing 4–5 genes on either side of a paralogue (C4B63_149g20) of TcCLB.506925.300. This locus is on Chr22-S in CL Brener. The alignments were manually examined and the reads-per-kilobase-per-million (RPKM) calculated for each of the contig in order to compare gene copy numbers. Depending on the library ~ 10–20% of the reads did not align. Chromosomal number was estimated in each sample using two different approaches: 1) Normalization of the mean read coverage for each chromosome by dividing by the median of all 43 chromosomes and multiply by 2; 2) Normalization of the RPKM (from Geneious) for each gene by dividing by the median for all genes and multiplying by 2 to generate the “copy number” of each gene, allowing to calculate the copy number mean for all genes on each chromosome.

Western blot. Cell were collected and washed twice with PBS and lysed with RIPA lysis buffer (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitor cocktail (Roche Diagnostics, Burgess Hill, UK). Total protein concentration was measured with Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Total protein lysates were mixed with SDS-PAGE Protein Loading Buffer Blue (National Diagnostics, Atlanta, GA), and boiled for 5 min. Proteins were separated in 15% polyacrylamide gels and transferred to a polyvinylidene difluoride (PVDF) or nitrocellulose membranes. The membranes were blocked with 5% skimmed milk containing Tween 20 (0.5%) for 1 h. The membranes were probed with primary antibodies at 4°C overnight followed by the incubation with the corresponding secondary antibodies labeled with horseradish-peroxidase for 90 min. The bound antibodies were detected by enhanced chemiluminescence reagents (Millipore, Burlington, MA, USA). Visualization of the transferred protein was done with FluoroChem HD2 (Protein Simple, CA, USA).

Histopathology. Heart tissues from mice were fixed in 10% buffered formalin. Tissue sections (5 µm) were stained with Haematoxylin-Eosin (H&E) and examined under light microscope for parasites. Images
were taken using a LEICA DMi1 using the LAS V4.12 software.

**Explant culture.** Explants of organs from *T. cruzi*-infected mice were cultured in LDNT medium. Briefly, organs were collected and excised into smaller pieces and put into 25 cm² non-vented flask containing 10 ml LDNT medium. Cultures were incubated at 26°C and examined twice a week for any growth of parasites for up to 8 weeks.

**Determination of IgG2a in blood serum by antibody ELISA.** For analysis of IgG2a, *T. cruzi* antigen was prepared by lysis of whole cells through rapid freeze-thaw technique. 96-well microplates were coated with *T. cruzi* antigen (5 µg/ml) at 4°C overnight using antigen coating buffer (phosphate-buffered saline, pH 9.0). Total IgG2a antibodies against *T. cruzi* were measured from blood of uninfected and infected mice. The antibody concentrations were measured at a 405 nm wavelength using SpectraMax microplate reader and data were analyzed by Softmax Pro Software (Molecular Devices LLC, Sunnyvale, CA, USA).

**T-cell proliferation and cytokine determination.** Splenocytes were harvested from wild type and STAT1 or STAT4 knock out mice. The cells were plated at a concentration of 5 · 10⁶ cells /ml in RPMI 1640 medium supplemented with 10% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin, and 1% HEPES. After stimulation of cells with 20 µg/ml *T. cruzi* freeze-thaw whole-cell antigen for 72 h, supernatants were collected, and the production of cytokines were measured by sandwich enzyme-linked immunosorbent assay (ELISA). Cytokines were analyzed according to the manufacturer's protocol using capture and detection antibody (BioLegend, San Diego, CA, USA). Cytokine concentrations were measured at a 405 nm wavelength using SpectraMax microplate reader and data were analyzed by Softmax Pro Software (Molecular Devices LLC, Sunnyvale, CA, USA).

**Complement-mediated killing assay.** Normal human serum was purchased from Sigma-Aldrich. The stationary-growth phase of *T. cruzi* culture was collected and washed twice with phosphate buffered saline. The cells were mixed with normal human serum at a final 30% concentration, and incubated at 37°C for 30 min with intermittent shaking in between. The cells were centrifuged at 1500 rpm for 5 min and kept at room temperature for 60 min so metacyclic parasites emerge from the pellet into the supernatant. The supernatant was collected, washed five times with phosphate buffered saline and the number of metacyclics were counted using improved Neubauer counting chambers.

**Scanning Electron Microscopy.** Cells were fixed and processed for SEM at the Campus Microscopy and Imaging Facility (CMIF), The Ohio State University, OH, USA. SEM images were obtained with a FEI Nova NanoSEM 400 Scanning Electron Microscope equipped with secondary and low-vacuum detector with a field-emission gun (FEG) electron source.

In vitro infections. All in vitro infections were performed in H9C2 and RAW cells. Host cells were plated at a concentration of 1 · 10⁵ cells/well in 12-well plates containing DMEM medium containing 10% HIFBS. Infection of H9C2 cells was performed using serum selected metacyclic-trypomastigotes and incubated at 37°C. After 24 h, the cells were washed with PBS in order to remove any extracellular trypomastigotes.
The plates were re-incubated in complete medium at 37°C 1–2 weeks. The formation of amastigotes and trypomastigotes were observed up to 2–3 weeks under light inverted microscope.

Animal infections. In order to examine the infectivity of *T. cruzi* wild type (WT) and Cyp19−/− DKO (double allele knock out) mutant parasites, AJ, STAT1−/−(BALB/c), and STAT4−/−(BALB/c) mice (Jackson Laboratories, Bar Harbor, ME, USA) were infected with 1 · 10^5 serum-selected trypomastigotes in 100 µl PBS through intraperitoneal route. Mice were examined daily for clinical symptoms to determine overall survival. All of the mice were examined for parasitemia by collecting blood from the tail vein twice a week. The survival days was counted from the day of inoculation until mice died. The statistically significant difference in survival between WT and DKO was calculated by Kaplan Meier survival analysis.

**Immunization and challenge infection in mice.** Mouse immunization was performed with serum-selected Cyp19 knock out trypomastigotes in AJ mice. Mice were immunized intraperitoneally four times with 1 · 10^5 trypomastigotes at 0, 4, 8, and 14 weeks. At 18 week, mice were challenged with 1 · 10^5 *T. cruzi* wildtype metacyclic trypomastigotes. Control AJ mice were infected with 1·10^5 serum-selected *T. cruzi* wild type trypomastigotes. All of the control mice died after 3 weeks of infection whereas immunized mice survived without developing any clinical illness (specifically weight loss, huddling behavior, decreased motility and shaking). All of the immunized and challenged mice were harvested at 34 week to evaluate further including explant culture.

**Statistical Analysis.** All data are expressed as mean ± SD. All statistical analyses were done in GraphPad Prism software. A student *t* test was used to determine statistical significance of differences among the groups. A *P* value of <0.05 was considered significant and indicated with asterisk.

**Results**

Generation of a Cyp19 knock-out parasite line. Since the published genome sequence of *T. cruzi* (CL Brener)\(^32\) indicated that cyclophilin 19 is expressed from a single copy gene \(^33,34\) we opted to create a Cyp19 null mutant using double allelic gene replacement (Fig. 1A). Plasmid constructs were engineered to contain 5'- and 3-UTRs of Cyp19 flanking sequences surrounding drug resistance genes for neomycin- and hygromycin phospho-transferases (NEO and HYG, respectively). These were amplified by PCR, cloned into pET15b plasmid, linearized and transfected separately into Brazil strain epimastigotes and selected using neomycin and hygromycin, respectively. Drug resistant parasites were cloned using limited dilution and examined for appropriate chromosomal integration of drug resistant constructs using PCR. A second allelic ablation was performed from a Neo-resistant clone with another round of transfection using a HYG-resistance marker. After double drug selection and limited dilution cloning, parasites were analyzed for appropriate integration by PCR for integration of transgenes, Cyp19 and flanking sequences (Fig. 1B). Western blot analysis of doubly resistant parasites confirmed loss of Cyp19 protein expression (Fig. 1C).

Whole nuclear genome sequencing of wild-type and Cyp19 knock-out strains. In order to verify the removal of Cyp19 genes and the appropriate integration of drug markers we performed whole genome
sequencing of several knock-out clones (denoted as SKO-NEO and -HYG, DKO-D0, -D11, -D12 in Figs S1-S4) and two WT parental strains. DKO-D0 was parasite line used in the vaccination studies in this paper. DKO lines D11 and D12 were recloned from the DKO-DO cloned line more than one year after continued growth in culture. The WT strains included parental WT strain used for the knock-out (termed WT-att), and an additional virulent WT strain (termed WT-vir) derived from WT-2 by multi-passage through immune-deficient STAT 4−/− mice. Recently the genome sequence of Brazil strain A4 was published which we used as a reference to compare of the genome sequences of our WT strains and the SKO and DKO mutant strains. This analysis confirmed the absence of two Cyp19 genes with NEO and HYG as expected (Fig. S1). Unexpectedly, we found that there was a preexisting trisomy of the central portion of chromosome 1, which contains another copy of the Cyp19 gene, in both the WT strains (shown for WT-att in Fig. S1 and for both in Fig. S2 and S3), indicating that the Cyp19 trisomy pre-existed in both WT parasite lines before transfection and is not as a result of compensation for loss of two Cyp19 gene copies. There is considerable aneuploidy in between the cell lines and there are increased copy number of several putative cyclophilin-like genes in the DKO lines in chromosomes 17 and 22 (Fig. S4). It is unclear whether these changes are a result of functional compensation for loss of two of the three Cyp19 genes in the DKO. The overall genomic features of the DKO lines is highly stable inasmuch as DKO-DO and DKO-D11, -12 are similar even after one year of continuous growth in culture (without drug pressure) without any repair of Cyp19 alleles by the remaining intact gene copy. Nevertheless, the loss of two Cyp19 genes results in attenuation of pathogenesis of in the DKO strain (see sections below). We have subsequently reanalyzed the DKO clones by Western blotting for the expression of Cyp19 from the remaining gene Cyp19 and have found intermittent expression of low levels of Cyp19 in from the DKO lines (not shown). Sequence analysis indicates that this additional copy is it identical to the other Cyp19 copies. We do not yet understand the nature of the ability of the variable expression of Cyp19 from these cells. However, we have not seen a change in the biologic phenotype (see sections below) related to Cyp19 deletion. We have attempted to remove the remaining Cyp19 copy using both a third round of targeted integration of a third drug-resistance marker and using CRISPR/Cas9 technology and both have been unsuccessful (not shown and Pedroso, et al, unpublished). We surmise that complete removal of Cyp19 from T. cruzi is not achievable, as Cyp19 is an essential gene for parasite survival.

Parasite growth and infection studies in vitro. Epimastigotes of the Cyp19 null mutants (DKOs) grew slower and reached peak densities that are significantly lower than their wild-type controls (Fig. 2A). The proportion of metacyclic forms that arose spontaneously in culture appeared earlier in the course of growth in culture and are significantly more abundant than wild-type parasites, reaching approximately 85–90% of the population (Fig. 2A). Single knock-out (SKO) parasites reached approximately half the peak density of WT parasites and produced about 35–50% metacyclic forms during growth in culture (Fig. 2A). The metacyclics of Cyp19 null parasites are resistant to complement mediated killing using normal human serum, as those of wild-type parasites (not shown) and had morphology similar to wild-type metacyclic forms (Fig. 2B). We suspect that diminished Cyp19 expression in the knock-out parasite lines leads to faster differentiation of epimastigotes into metacyclic trypomastigotes resulting in the
higher proportion of metacyclics in the overall culture. Since metacyclic parasites do not replicate, this limit results in a reduced overall density of parasites in the culture.

In order to test the ability of SKO and DKO parasite lines to infect we used a rat heart myoblast (RHMs) cell line (H9C2), our prototype line for in vitro assessment of parasite infection, and incubated them with isolated metacyclic stages the DKO, SKO and WT parasite lines. Infection of RHMs with WT parasites results in the development of intracellular amastigotes by 2–3 days post-infection that increase in number, eventually transforming into motile tissue-culture tryomastiogotes (TCTs) which then exit host cells (Fig. 2C, upper panels). The course of infection of RHMs with SKO parasites is similar to that of WT parasites, although they appear to grow slower intra-cytoplasmically, but eventually reach similar densities and transform into TCTs that exit the cell (Fig. 3C, middle panels). Infection of RHMs with DKO parasites results in the production of scant amastigotes, which fail to replicate intracellularly and eventually degenerate (Fig. 3C, lower panels). Infections of RHMs with DKO parasites fail to give rise to any demonstrable TCTs. We have not observed amastigote or TCTs forms produced over prolonged observations of RHMs cultures incubated with DKO parasites for 1–2 months. We have repeated infectivity studies with the DKO line at least 10 times with the same results. Additional DKO clones, D-11 and D-12, obtained by recloning the DKO line after one year on continuous culture, were repeatedly tested for their ability to infect and replicate in H9C2 cells. Neither parasite line was able to infect these cells or produce TCTs (not shown) as we have seen with the original DKO parasite line. We compared the ability of WT and DKO parasites to cause productive infection in phagocytic RAW cells (Fig. 3). As expected infection with WT metacyclics led to the development of amastigotes and formation and release of extracellular trypomastigotes (Fig. 3 lower panel). In contrast, infection of cells with DKO metacyclics did not lead to a productive infection with either the development of replicating intracellular amastigote or the production of extracellular trypomastigotes.

Infection studies on vivo. In order to test the effect of the loss of Cyp19 on parasite virulence in animals we used immunocompetent AJ mice (males 4–6 weeks of age) infected with purified metacyclics of the either WT or DKO parasites (10^5 parasites per IP inoculation). Control infections with WT parasites consistently results in the onset of clinical infection (shivering, ruffled coat, huddling behavior and diminished appetite and activity) within 1-1.5 weeks, and death within 3–4 weeks, post-infection (Fig. 4A). These mice reach a peak density of parasitemia just prior to death or sacrifice (approximately 45–70 parasites per 200 hfps/mouse) (not shown). Infection of animals with DKO parasites does not result in the development of clinical disease and 100% of the mice survive infection (Fig. 4A). Over the course of four separate experiments, we have not observed clinical signs of infection and all mice survived and remained healthy up to 15 weeks post-infection with DKO parasites. We have not observed clinical infection of DKO-infected mice over prolonged observation of up to one year. We could not detect parasitemia in weekly microscopic analysis of the blood from DKO-infected. Analysis of blood and tissues (liver, spleen, hearts, GI mesentery, stomach and large intestine) from infected animals using both tissue explantation culture and histopathology demonstrated abundant parasites in most tissues from WT-infected animals whereas we could not find parasites in DKO-infected animals (Fig. 4B and C).
cruzi-specific PCR analysis of selected tissues of DKO-infected animals did produce signals consistent with the presence of parasite DNA (not shown). In the absence of culturable parasites from tissue, this likely represent the presence in the tissues of lingering DNA from the inoculum used for infection.

Mice infected with DKO parasites one year after were treated with dexamethasone (5 mg/kg) in order to test whether induction of immunosuppression would induce clinical infection from latent infection. Thirty days of treatment of DKO-infected animals with dexamethasone neither induced clinical infection. Further, parasites were not detected by either explantation or histopathologic analysis of blood and tissues from dexamethasone treated DKO-infected animals. These results indicated that DKO-mutants do not lead to acute, chronic or latent infection of immunocompetent mice.

Cyp19-depleted parasites do not cause infection in immuno-deficient mouse strains. Infection of immuno-deficient STAT-1−/− or STAT-4−/− mice, which are hyper-susceptible to *T. cruzi* infection were also tested the DKO for pathogenicity. As expected, infection of either STAT-1−/− or STAT-4−/− mice with WT parasites resulted in high levels of parasitemia and death of 100% of the mice between 15–20 days post infection (for STAT-1−/−) or 30–45 days (Fig. 5A) for STAT-4−/− mice (Fig. 5B). Infection with the DKO parasites, surprisingly, resulted in no parasitemia nor clinical symptoms or death in either immuno-deficient mouse strain. We have thrice repeated these experiments with the same result and have observed DKO infected mice for 6 months (for STAT-4−/− infected) and 3 months for (STAT-1−/− infected) mice strains. Analysis of the heart tissue of STAT-4 infected animals (Fig. 5C) shows abundant parasite nests in the hearts of animals infected with WT parasites, however the hearts of DKO-infected mice were free of parasites or inflammatory infiltrates, indicating that DKO parasites did not infect or cause damage to this important end-organ for Chagas disease.

DKO parasites induce anti-*T. cruzi* immunity which protects against acute Chagas disease. Because DKO parasites do not infect cells *in vitro* or mice, we tested the ability of repeated infections of DKO parasites to produce an anti-*T. cruzi* immune responses. Repeated inoculation of animals with WT produces parasite-specific IgG2a levels to rise reaching a peak at near the time of death (Fig. 6, left panel). Repeated IP inoculation of immunocompetent AJ mice with 10^5 parasites also did not result in clinical disease or death over multiple experiments. However, the level of parasite-specific IgG2a increasingly rose with repeated injection of DKOs, indicating the development of B-cell anti-parasite immunity. Splenic cells harvested from DKO “immunized” animals produced high levels of IFN-γ and lower levels of IL-4 and IL-10 when stimulated with parasite antigen indicating the development of Th1-cell immunity (Fig. 6B). We tested whether the anti-parasite immunity developed by repeated injection was able to protect mice from development of disease or death from infection by WT parasites (Fig. 7). Infection of mice with WT parasites, as expected, resulted in the onset of clinical infection and death between 3–4 weeks post-infection (Fig. 7A and B). Challenge of mice previously “immunized” with repeated injections of DKO parasites with WT parasites did not develop clinical disease and are completely healthy up to 34 weeks post-challenge (Fig. 7B). We found no evidence of parasite infection in DKO immunized WT challenged animals (Fig. 7D). We have repeated this experiment 3 times with the same results. In order to test
whether latent infection existed in DKO-immunized WT challenged animals we treated animals with dexamethasone approximately one year post-infection. After 60 days of dexamethasone treatment, we observed no clinical infection (Fig. 7B) or the presence of parasites in explantation or histopathologic analyses (not shown).

A single immunization with DKO parasites is sufficient for protective immunity. Our experiments up to this point indicated that four doses of DKO parasites given over a span of 4.5 months induced effective protective immunity to WT challenge. We tested whether a single dose of the DKO vaccine could elicit protection (Fig. 8). DKO administered as described previously and animals were challenged 6 months after immunization. Observation of immunized animals over 6 months post-infection resulted in no evidence of clinical infection (Fig. 8B) whereas, as expected, 100% of non-immunized animals died within 21–25 days post challenge (Fig. 8A and B). Explantation and histopathologic analyses indicated the presence of parasites only in the non-immunized challenged animals (Fig. 8C and D).

Discussion

This report describes the engineering of Trypanosoma cruzi in the genes encoding cyclophilin 19, an enzyme which catalyzes cis-trans peptidyl-prolyl isomerization of proteins (PPIases). Our previous studies of this enzyme fortuitously found that it was secreted by epimastigotes and important in the neutralization of anti-parasiticidal proline-containing insect anti-microbial peptides. As a group, PPIases are expressed by organisms from all Kingdoms where they serve myriad functions including acting as a protein chaperone (folding of nascent proteins and re-folding of aggregated proteins), in ROS production and scavenging, gene expression and RISC complex formation and miRNA regulation. Cyp19 is the only T. cruzi PPIase that has been reported to be secreted, which portends a role in interacting with extracellular substrates. We found that Cyp19 is expressed in and released extracellularly by all life-stages of the parasite (Pedroso, et al, unpublished), suggesting in each parasite stage it may function to engage and modify different host target proteins. In fact, our recent data indicated that secreted Cyp19 induces the intracellular ROS production in host cells which is critical for amastigote replication (Pedroso, et al, unpublished).

The double-knock-out (DKO) mutant parasite line with diminished Cyp19 expression is viable in culture, but has a diminished growth rate, and is defective in its ability to reach a peak density of that of wild type parasites, suggesting a defect in replication as epimastigotes. The mutant line differentiates rapidly into metacyclic trypomastigotes, further suggesting a defect in normal cell cycle control. We surmise that the rapid conversion of epimastigotes to metacyclic-forms at early points in cultivation of cells contributes to the inability of these cells to reach higher densities, since metacyclic forms do not undergo replication. The replication of low numbers or epimastigotes prior to their differentiation into metacyclic form is the key to their continued survival in culture. The DKO metacyclics are resistant to complement-mediated killing by normal human serum and are morphologically similar to those of wild-type parasites, suggesting the decreased Cyp19 expression does not affect these properties. When used to infect mammalian host cells, metacyclic parasites enter cells at a reduced rate and form scant amastigotes-like
structures, which degenerate and disappear from host cells. DKO parasites do not lead to productive infection and fail to give rise to cell-derived trypomastigotes (TCTs). Prolonged observation of DKO infected cultures, up to several weeks, fail to show replication of additional amastigote and of the production of TCTs, indicating profound defect in their ability to replicate and differentiate. Since Cyp19 is likely involved in the maturation and expression of other parasite proteins, its absence likely affects a multitude of pathways, including both constitutive house-keeping proteins (e.g. cell cycle machinery, differentiation, etc.) as well as host involved in parasite infectivity. Thus, the DKO line probably has multiple defects in several hitherto unknown pathways.

Comparison of the whole genome sequences of single- and double-knockouts with wild-type lines indicate the presence of pre-existing trisomy in chromosome 1 in Brazil strain, which contains the genes for cyclophilin 19. This was unexpected since in the published genomes of *T. cruzi* have indicated that this gene is present as single copy on two alleles. Our attempts to create a cyclophilin 19 null mutant using either CRISPR/Cas9 technology in the DKO or wild-type parasites, or removal of the last Cyp19 gene copy in the DKO using an additional allelic replacement has been unsuccessful, suggesting that complete removal of cyclophilin 19 may not be possible, probably because it is indispensable for parasite growth and survival. We have found that DKO parasites intermittently express small levels of Cyp19 protein detectable by Western blotting. We recloned the initial DKO population, after over one year in culture, and analyzed these by whole genome sequencing. We found that the genetic structures stable after continuous culture and reanalysis of these by Western blotting also indicated they intermittently expressed Cyp19 protein. These clones, like the original DKO, are unable to infect cells *in vitro* or mice. Interestingly, we did see selected trisomy in other chromosomes in the DKO (chr 17 and 22) (Fig. S3) each of which contains cyclophilin-like genes. In the absence of reversion of attenuation, it is possible that increases in these genes could compensate for loss of cyclophilin 19 genes for other functions unrelated to virulence. These results indicate that the attenuated phenotype is highly stable. Whether there are other defects (small deletions, SNPs, etc) in the DKO clones, other than diminished Cyp19 expression, to account for their profound attenuation is unknown.

Infection of mice with DKO parasites does not lead to the development of clinical disease or death for up to one year of observation. Analysis of tissues from infected animals shows no detectable viable parasites using histopathology or culture of explanted tissues, including those from GI mesentery, heart, spleen, or liver, indicating the absence of deposition of or replication of parasites in the tissue of DKO infected animals. Parasite DNA was detected by parasite-specific PCR analysis of some tissues (not shown) indicating that the inoculum of DKO circulates and deposits in tissues but does not survive and grow. Even repeated infection of mice with $10^5$ DKO parasites fails to cause disease and does not affect host survival. These results clearly indicate that the DKO line is highly attenuated from causing disease *in vivo*. This likely relates to the inability of the DKO to infect, grow and survive in host cells, as we saw in the *in vitro* studies using various cell lines. The attenuated phenotype is highly stable, since infection studies with the initial DKO line and recloned lines after one year in culture were also unable to infect host cell lines in vitro or mice. This correlated with the stability of the genetics of the DKO lines as seen in our
comparative whole genome analysis. The stability of the attenuated phenotype is important especially since this line may be a promising live vaccine strain for Chagas disease. Fortuitously repeated infection of mice with the DKO line leads to increasing levels of anti-\textit{T. cruzi} T- and B-cell immune responses. Splenocytes harvested from these animals produce high levels of IFN-\(\gamma\), IL-12 and low levels of IL-4 and IL-10 indicative of a Th-1 response, which has been reported to advantageous to the clearance of parasites \(^{15,16}\). Challenge of “DKO-immunized” with high-dose virulent wild-type Brazil strain \textit{T. cruzi} metacyclics failed to develop clinical signs of infection disease and had 100% survival whereas infected non-immunized animals had 100% mortality between 21–30 days post-infection. This indicates the anti-\textit{T. cruzi} immunity elicited by the DKO-parasite immunization was 100% effective at protecting animals from acute Chagas disease. Surprisingly, a single immunization with the DKO line was effective at providing completely protection to wild-type parasite challenge 6 months after inoculation. This indicates that the immune response elicited by the DKO vaccine strain is highly immunogenic and long-lasting. An advantage of the DKO mutant is that it provides exposure of the immune response to a large array of metacyclic stage antigens which may give rise to greater immunity than would subunit vaccines composed of a single of few antigens. In addition, the using the whole parasite might serve as a natural adjuvant, naturally bolstering the immune response. We have not found any deleterious effects from administration of the DKO vaccine strain to animals. This is a concern since the development of host immunity to certain parasite antigens can serve to generate autoimmunity through molecular mimicry and this could lead to tissue inflammation \(^{42}\). Our safety studies included using STAT-1 and STAT-4 deficient mouse strains to test the DKO. The mutant parasite line was unable to infect these mutant mouse strains, indicating that even in immune-deficient mice the DKO line is unable to replicate. Induction of immunosuppression using dexamethasone, in immunocompetent mice infected with DKO parasites did not result in re-crudeced infection, indicating that the DKO strain does not cause smoldering latent infection. Further, dexamethasone treatment of immunized, WT-challenged animals did not result in emergence of latent infection. This is highly important since it suggests that the immunity induced by the DKO vaccine results in sterile immunity not allowing wild-type parasites to reach or growth within target tissues.

Currently, there are no approved vaccines for the prevention or treatment of Chagas disease. There are several reports of Chagas vaccines in development including those based on recombinant protein subunits \(^{43,44,45}\), naked plasmid DNA-protein expressing constructs \(^{46}\), peptides \(^{47}\), adenoviral and \textit{Salmonella} vector systems for expression of parasite proteins and several live attenuated \textit{T. cruzi} vaccines and a live \textit{T. rangeli} vaccine \(^{48,49,50,51}\). Several mutant parasite lines have been tested as live vaccine strains; either those attenuated by long-term in vitro cultivation \(^{52}\) or those engineered with specific genetic defects resulting in diminished growth in culture and reduced virulence in animals but are able to provoke anti-\textit{T. cruzi} immunity and partially reduce the magnitude of infection of animals when challenged with the parental wild-type strain \(^{53,54,55}\).

Our results demonstrate that the DKO mutant described here is highly effective live parasite vaccine for prevention of acute Chagas disease in the mouse model. It is completely effective in a single dose and it
provides sterile immunity and is safe in immune-deficient hosts. Other potential roles of this strain including using this as a heterologous antigen delivery system. Further studies with this strain include its ability to cross-protect against infection by other *T. cruzi* strains, the length of protective immunity.

**Declarations**

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**Author contributions:** BKJ, SV, NB, JM, AS and GP participated in conducting experiments and compiling data; PJM, SS, ARS and BSM designed experiments and analyzed the data; BKJ and BSM wrote the manuscript; all authors participated in editing the manuscript.

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