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Supplementary Materials for

A modified drug regimen clears active and dormant trypanosomes in mouse models of Chagas disease

Juan M. Bustamante, Fernando Sanchez-Valdez, Angel M. Padilla, Brooke White, Wei Wang, Rick L. Tarleton*

*Corresponding author. Email: tarleton@uga.edu

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Fig. S3. Parasites recovered from mice treated for 55 weeks with 100 mg/kg of BNZ (100w/STOP) had unchanged susceptibility to BNZ in vitro.
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Other Supplementary Material for this manuscript includes the following:

(available at stm.sciencemag.org/cgi/content/full/12/567/eabb7656/DC1)

Movie S1 (.mov format). Nearly unrestricted expansion of *T. cruzi* amastigote nests in heart and skeletal muscle in IFN-γ–deficient mice.
Movie S2 (.mov format). Uncontrolled *T. cruzi* development in IFN-γ–deficient mice.
Movie S3 (.mov format). Controlled *T. cruzi* amastigote nests in infected C57BL/6 wild-type mice.
Movie S4 (.mov format). Partial clearance of *T. cruzi* parasites following brief BNZ treatment.

Movie S5 (.mov format). Multicolor light sheet microscopy reveals active and dormant parasite clearance following weekly BNZ treatment.
Raw data file. Raw data file.xlsx.

Supplementary Materials

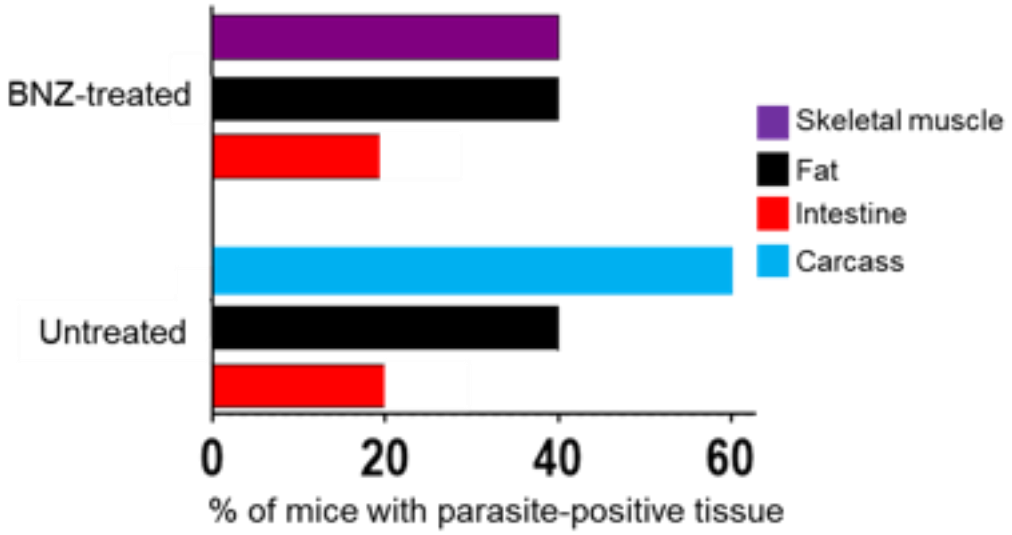


Fig. S1. Tissue persistence of ARC-0704 *T. cruzi* strain pre- and post-full course BNZ treatment in chronically infected mice. Cohorts of ten C57BL/6 wild-type mice were infected with 10^4 wild-type trypomastigotes from ARC-0704 *T. cruzi* strain. Five mice were treated with 100 mg/kg of BNZ on days 75 to 115 post-infection and 5 mice were left untreated. Two weeks after the end of treatment, mice were euthanized, perfused and tissues were collected. DNA was extracted from cardiac and skeletal muscle, gastrointestinal tract, brain, spinal cord, adipose tissue and the complete mice carcass and parasite loads were assessed by qPCR. Data shown only for tissues that were positive in at least one animal. Data indicates the percentage of mice that presented a tissue positive signal in untreated and treated animals.

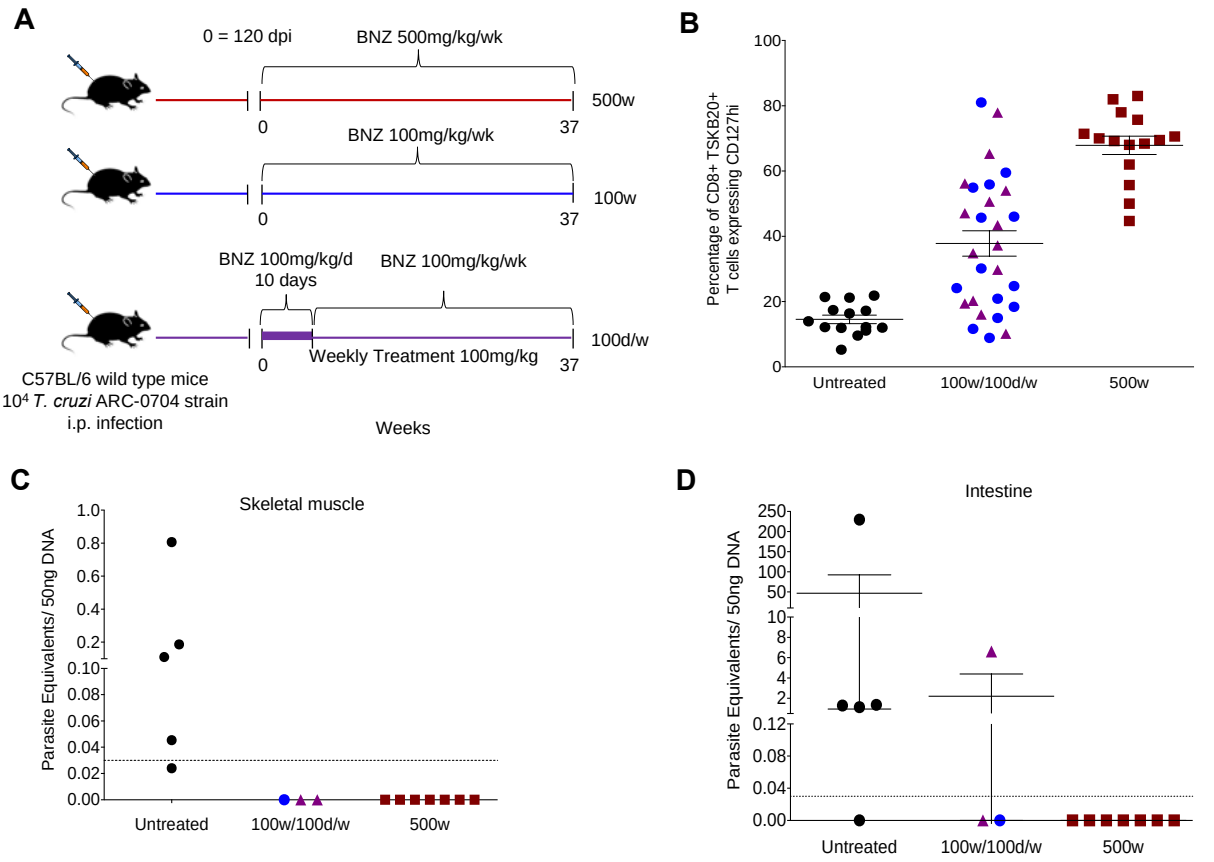


Fig. S2. BNZ given weekly over 37 weeks cures mice with chronic *T. cruzi* infections. (A) Schematic representation of infection, treatment and assessment of treatment efficacy corresponding to the experiment described in Figure 2. C57BL/6 wild-type mice (14-15 mice in each group) were infected intraperitoneally with 10^4 trypomastigotes of the ARC-0704 strain of *T. cruzi* and left untreated or treated weekly, starting at 120 days post-infection, with BNZ at 100 or 500mg/kg concentration for 37 weeks. One group was initially treated with BNZ at 100mg/kg for 10 consecutive days followed by a weekly treatment with BNZ at the same concentration of 100mg/kg. **(B)** Expression of the memory maintenance marker CD127 in blood on CD8+ TSKB20-tetramer+ T cells from individual mice untreated or treated at 37 weeks of treatment. **(C-D)** *T. cruzi* DNA isolated from **(C)** skeletal muscle and **(D)** intestine of untreated or treated (animals expressing a relatively high frequency of CD127) mice at 37 weeks and assayed by quantitative real time polymerase chain reaction. Blood from these mice collected at 37 weeks of treatment was submitted to hemoculture assays and analyzed for parasite growth for >60 days.

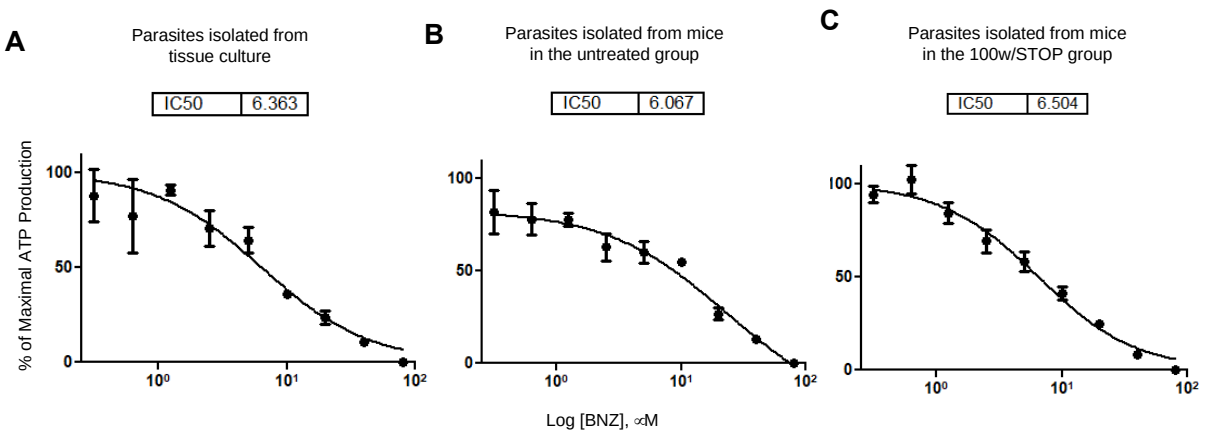


Fig. S3. Parasites recovered from mice treated for 55 weeks with 100 mg/kg of BNZ (100w/STOP) had unchanged susceptibility to BNZ in vitro. IC_{50} curves for *T. cruzi* epimastigotes of the ARC-0704 strain from tissue culture (A) or hemocultures obtained at 77 weeks from mice untreated (B) or treated with 100mg/kg of BNZ for 55 weeks (C). IC_{50} was determined as the drug concentration that was required to inhibit 50% of ATP production compared to that of parasites with no drug exposure (see Material and Methods).

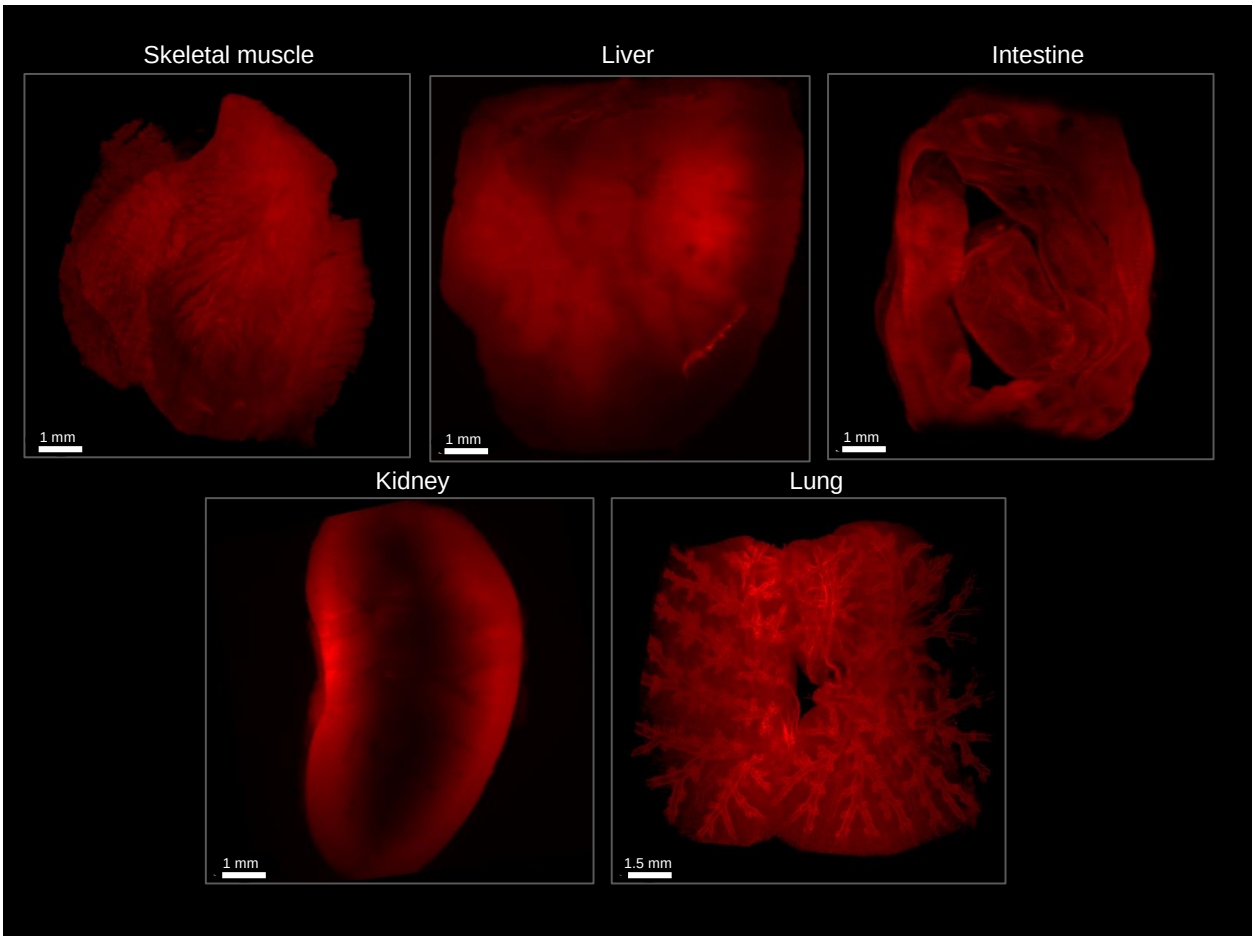
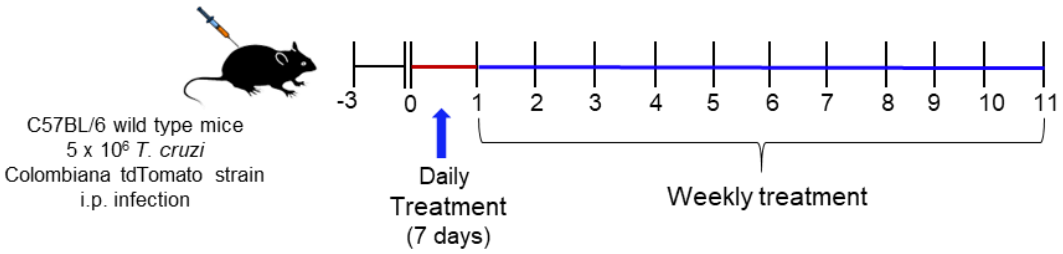


Fig. S4. 3D reconstruction of whole organs from uninfected C57BL/6 mice. A two-month old naive C57BL/6 wild-type mouse was euthanized, perfused and the organs were clarified and prepared for LSFM. The tissue autofluorescence (red background) in the 532 nm channel allows for the visualization of the entire organ morphology.

A



B

		Weeks						
		0	1	3	5	7	9	11
BNZ 100mg/kg	Heart	36	0	1	0	4	1	0
	Muscle	64	2	6	4	5	5	6
	Intestine	26	2	8	6	7	3	3
BNZ 500mg/kg	Heart	48	0	3	0	0	0	0
	Muscle	50	0	1	4	0	0	0
	Intestine	30	4	6	3	0	0	0

Fig. S5. *T. cruzi* clearance as assessed by whole-organ LSFM. (A) Schematic representation of infection and treatment. C57BL/6 wild-type mice were intraperitoneally infected with 5 x10^6 trypomastigotes of tdTomato-expressing Colombiana strain of *T. cruzi*, treated with 100mg/kg of BNZ daily for 7 days, starting at 3 days post-infection, and then weekly at 100 or 500mg/kg concentration over 10 weeks. One mouse per timepoint was sacrificed and perfused. Heart, muscle and large intestine were collected, clarified and scanned by LSFM at weeks 0, 1, 3, 5, 7, 9 and 11. **(B)** Automated quantification of total *T. cruzi* amastigote nests in 3D reconstructions.

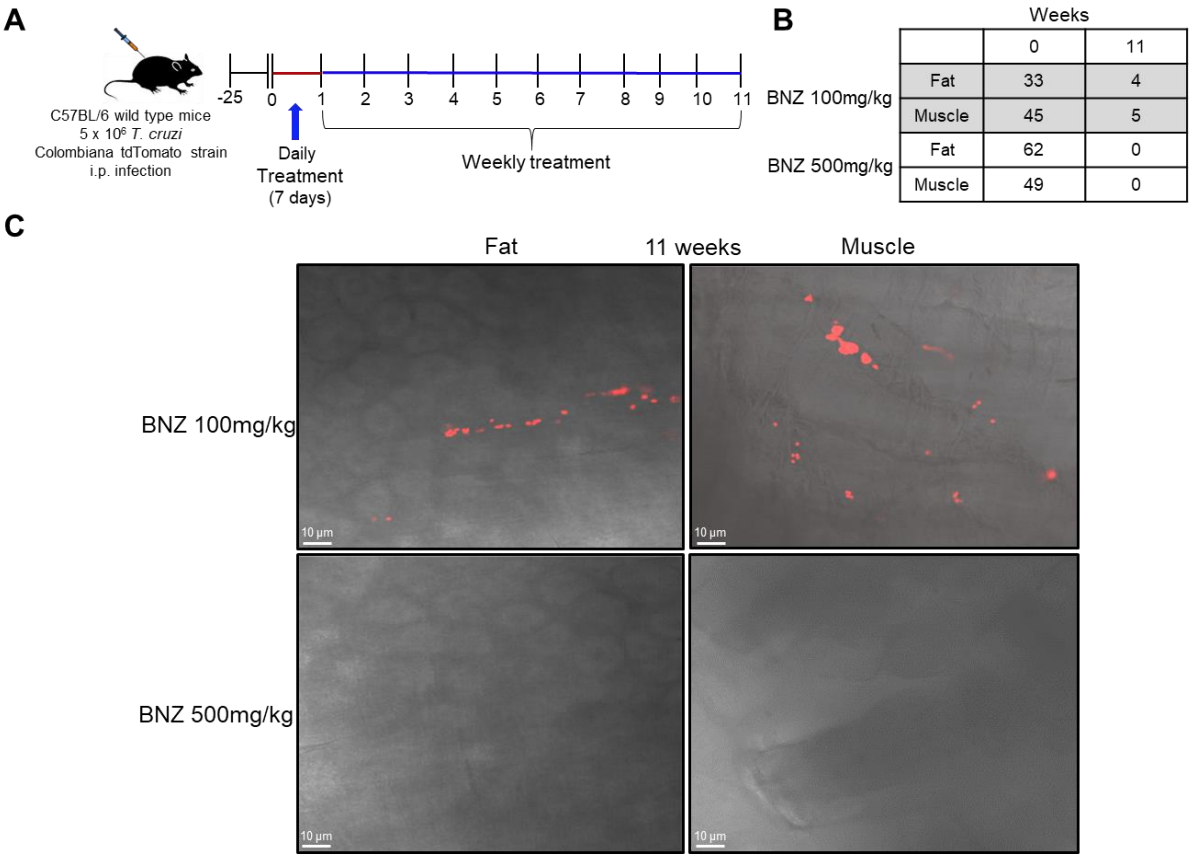


Fig. S6. Drug dose–dependent differential clearance of an established *T. cruzi* infection as assessed by confocal microscopy. (A) Schematic representation of infection and treatment. C57BL/6 wild-type mice (2 mice per group) infected 25 days previously with 5 x10^6 tdTomato-expressing Colombiana trypomastigotes of *T. cruzi* were treated with 100mg/kg of BNZ daily for 7 days, and then weekly at 100 or 500mg/kg concentration for 10 weeks. Imaging assessment of treatment efficacy was carried out at weeks 0 (pretreatment) and 11. Mice were euthanized and peritoneal adipose tissue and skeletal muscle were prepared on 35 mm glass-bottom petri dishes for CLSM. **(B)** Quantification of total *T. cruzi* amastigote nests of mice treated with 100 or 500mg/kg of BNZ. **(C)** Representative CLSM images showing amastigote nests in adipose and skeletal muscle tissue of mice at 11 weeks. Skeletal muscle samples were sliced in 0.5 mm sections and exhaustively scanned by CLSM. The peritoneal adipose tissue sample from each mouse is ~0.3 g of tissue spread over a surface of ~1.6 cm^2 when mounted for CLSM analysis.

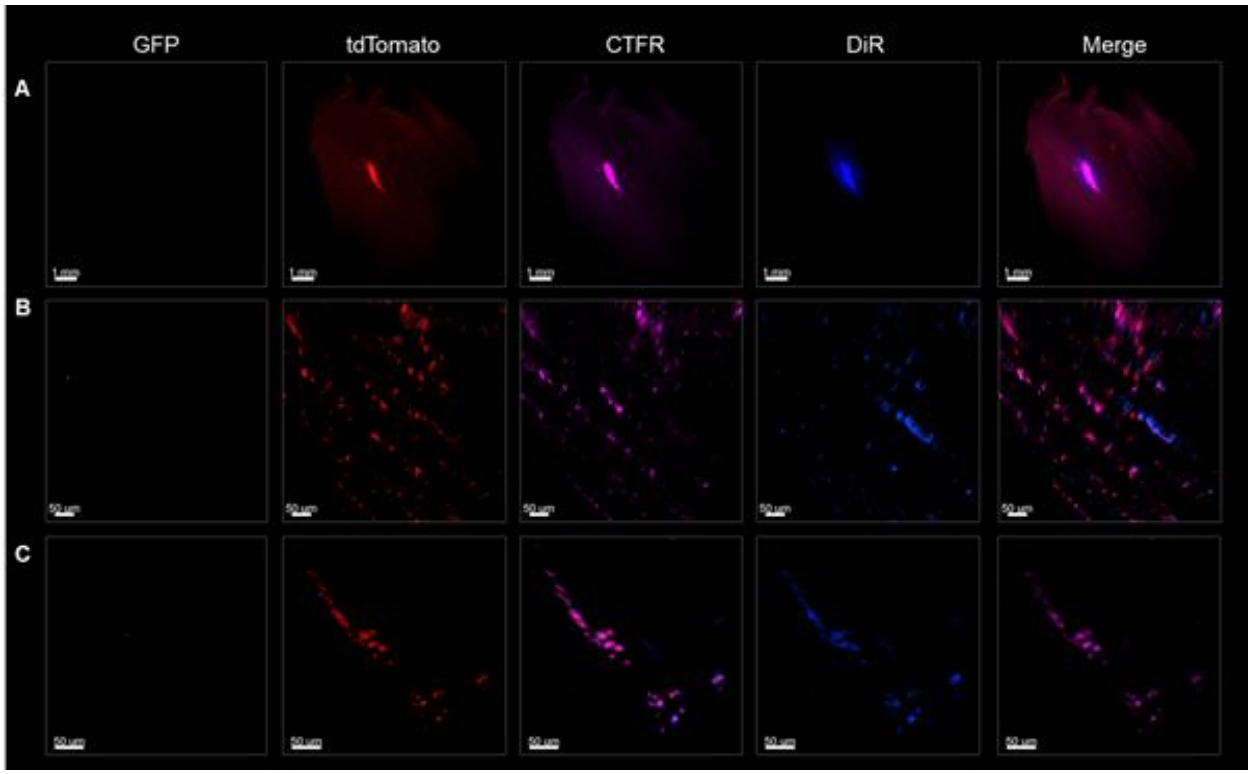


Fig. S7. CUBIC tissue clarification protocols preserve fluorescence in dye-stained parasites. C57BL/6 wild-type mice were intramuscularly infected with 5 x10^5 trypomastigotes of tdTomato-expressing Colombiana strain of *T. cruzi* stained with DiR near-infrared and CellTrace Far Red (CTFR) fluorescent dyes. Twenty-four hours post-infection, mice were euthanized, perfused and skeletal muscle in the area of infection was dissected, clarified and scanned by LSFM. **(A)** Muscle 3D reconstruction reveals tdTomato (red), CTFR (purple) and DiR (blue)-positive signals after tissue clarification. GFP fluorescence (green) was used as control to reveal nonspecific signal (artifacts). Stained parasites were primarily concentrated on the site of injection. **(B and C)** Representative single optical slices from the 3D reconstruction showed in (A) depicting tdTomato, CTFR and DiR-positive amastigotes. Tissue clarification protocol does not quench dye-stained amastigotes.

Table S1. Automated quantification of total tdTomato-positive amastigote nests or total DiR-positive dormant parasites in tissues of untreated and treated mice with 500 mg/kg of BNZ.

(A) Automated counting of total tdTomato-positive amastigote nests in heart, muscle and intestine of untreated and treated mice with 500mg/kg of BNZ. Tissues samples from 3 different animals (indicated as M1/M2/M3) were analyzed at each timepoint. **(B)** Automated quantification of total DiR-positive (dormant) parasites in tissues of untreated and treated mice with 500mg/kg of BNZ. Avg.= average of the total amount of amastigote nests or dormant parasites from three tissue samples. BNZ = benznidazole. SD = standard deviation.

A Proliferating parasites

		0 (weeks)			3 (weeks)			6 (weeks)			9 (weeks)			13 (weeks)		
		M1/M2/M3	Avg.	SD	M1/M2/M3	Avg.	SD	M1/M2/M3	Avg.	SD	M1/M2/M3	Avg.	SD	M1/M2/M3	Avg.	SD
Untreated	Heart	3/3/2	2.6	0.4	1/0/0	0.3	0.4	0/0/0	0	0	1/1/1	1	0	0/0/0	0	0
	Muscle	4/50/20	24.6	19	6/35/40	27	14.9	4/19/10	11	6.1	2/5/0	2.3	2	0/2/1	1	0.8
	Intestine	2/6/2	3.3	1.8	4/1/2	2.3	1.2	11/5/2	6	3.7	1/3/4	2.6	1.2	1/3/2	2	0.8
BNZ 500mg/kg	Heart	2/4/6	4	1.6	0/0/0	0	0	0/0/0	0	0	0/0/0	0	0	0/0/0	0	0
	Muscle	12/43/32	29	12.8	3/5/4	4	0.8	0/0/0	0	0	0/0/0	0	0	0/0/0	0	0
	Intestine	7/9/5	7	1.6	3/4/1	2.6	1.2	0/0/0	0	0	0/0/0	0	0	0/0/0	0	0

B Dormant parasites

		0 (weeks)			3 (weeks)			6 (weeks)			9 (weeks)			13 (weeks)		
		M1/M2/M3	Avg.	SD	M1/M2/M3	Avg.	SD	M1/M2/M3	Avg.	SD	M1/M2/M3	Avg.	SD	M1/M2/M3	Avg.	SD
Untreated	Heart	1/3/0	1.3	1.2	4/0/3	2.3	1.6	0/0/0	0	0	2/0/0	0.6	0.9	0/0/0	0	0
	Muscle	1/5/3	3	1.6	0/0/17	5	8	1/0/1	0.6	0.4	0/2/0	0.6	0.9	1/1/1	1	0
	Intestine	13/12/9	11.3	1.6	2/5/7	4.6	2	4/1/ 2	2.3	1.2	0/0/0	0	0	1/0/1	0.6	0.4
BNZ 500mg/kg	Heart	6/9/14	9.6	3.2	0/0/0	0	0	0/0/0	0	0	0/0/0	0	0	0/0/0	0	0
	Muscle	2/1/4	2.3	1.2	2/2/3	2.3	0.4	0/0/0	0	0	0/0/0	0	0	0/0/0	0	0
	Intestine	6/9/10	8.3	1.6	2/9/6	5.6	2.8	0/0/0	0	0	0/0/0	0	0	0/0/0	0	0

Table S2. Automated quantification of total DiR-positive dormant parasites from the experiment performed in Fig. 5 and fig. S5. (A) Automated quantification of total DiR-positive (dormant) parasites from the experiment presented in Figure 5. C57BL/6 wild-mice were intraperitoneally infected with 4 x10^6 tdTomato-expressing Colombiana trypomastigotes of *T. cruzi* stained with DiR near-infrared dye and treated weekly for 7 weeks, with 100 or 500 mg/kg of BNZ, starting at 5 days post-infection. At each indicated time point, one mouse per group was sacrificed, perfused and heart and skeletal muscle were dissected, clarified and scanned using LSFM. **(B)** Automated quantification of total DiR-positive (dormant) parasites from the experiment described in Fig. S5. C57BL/6 wild-type mice were intraperitoneally infected with 5 x10^6 trypomastigotes of the tdTomato-expressing Colombiana strain stained with DiR near-infrared dye, treated with BNZ at 100mg/kg daily for 7 days, starting at 3 days post-infection, and then weekly at 100 or 500mg/kg concentration over 10 weeks. One mouse per time point was sacrificed and perfused. Heart, muscle and intestine were collected, clarified and scanned by LSFM at weeks 0, 1, 3, 5, 7, 9 and 11. BNZ = benznidazole. ND = not determined.

A

Dormant parasites

		0 (weeks)	2 (weeks)	4 (weeks)	6 (weeks)	8 (weeks)
BNZ 100mg/kg	Heart	23	ND	4	1	1
	Muscle	64	7	3	1	3
BNZ 500mg/kg	Heart	31	ND	1	0	0
	Muscle	45	6	4	0	0

B

Dormant parasites

		0 (weeks)	1 (weeks)	3 (weeks)	5 (weeks)	7 (weeks)	9 (weeks)	11 (weeks)
BNZ 100mg/kg	Heart	22	9	5	0	0	0	0
	Muscle	41	12	2	1	1	1	1
	Intestine	1	8	7	1	1	0	1
BNZ 500mg/kg	Heart	43	0	0	0	0	0	0
	Muscle	55	0	1	0	0	0	0
	Intestine	27	6	3	1	0	0	0

Movie S1. Nearly unrestricted expansion of *T. cruzi* amastigote nests in heart and skeletal muscle in IFN-γ–deficient mice. 3D reconstructions of CUBIC-clarified tissues of IFN-gamma deficient mice infected with 2 x10^5 tdTomato-expressing Colombiana trypomastigotes of *T. cruzi* at day 17 post-infection. A total of 1151 individual slices of the heart (top) and 559 of the skeletal muscle (bottom) were acquired via LSFM. Bright red amstigote "nests indicate muscle cells infected with *T. cruzi* . Zoom-ins of the 3D reconstructions (left) reveal infected cells at various stages of parasite development, including large, medium and small amastigotes nests as well as recently burst host cells (skeletal muscle, bottom left). Orthoslicing through the tissues shows parasite nests at various tissue depths. Comparable imaging results were achieved in 3 independent animals.

Movie S2. Uncontrolled *T. cruzi* development in IFN-γ–deficient mice. 3D visualization of a clarified section of the large intestine, liver, epididymis and heart from IFN-gamma deficient mice. A mouse was infected with 2 x10^5 *T. cruzi* Tdtomato-expressing Colombiana (red;right) or eGFP-expressing Brazil strains (green; left) and tissues processed at day 17 post-infection. A total of 368 individual slices of the large intestine, 602 of the liver, 529 of the epididymis and 2303 of the heart were imaged by LSFM Comparable imaging results were achieved in 2 animals.

Movie S3. Controlled *T. cruzi* amastigote nests in infected C57BL/6 wild-type mice. 3D visualization corresponding to clarified heart and skeletal muscle of wild-type C57BL/6 mice infected with 2 x10^5 tdTomato-expressing Colombiana trypomastigotes at day 17 post-infection. A total of 1200 individual slices of the heart and 378 of the skeletal muscle were acquired via LSFM. These findings were verified in at least 5 mice.

Movie S4. Partial clearance of *T. cruzi* parasites following brief BNZ treatment. 3D reconstructions of C57BL/6 wild-type mouse hearts infected with 2 x10^5 tdTomato-expressing Colombiana strain trypomastigotes of *T. cruzi*. Mice were left untreated or treated with 2 daily doses (on days 18 and 19 post infection) of BNZ at 100mg/kg concentration. At 20 dpi, mice were euthanized, perfused and the heart was clarified and prepared for LSFM. A total of 1966

and 2585 individual slices were acquired for the untreated and treated mice heart, respectively. This assay was a single experiment.

Movie S5. Multicolor light sheet microscopy reveals active and dormant parasite clearance following weekly BNZ treatment. Representative skeletal muscle reconstructions of C57BL/6 mice infected with 4 x10^6 DiR near-infrared dye-stained trypomastigotes of the tdTomato-expressing Colombiana strain of *T. cruzi*. Mice were left untreated or treated weekly, starting 37 days post-infection, with BNZ at 500mg/kg concentration over 12 weeks. On weeks 0, 3, 6, 9 and 13, mice from each group were euthanized and clarified tissues scanned by LSFM.. In untreated mice, a total of 711 individual slices were obtained for week 0 and 1252 for week 13. In treated mice, a total of 781 individual slices were obtained for week 0 and 945 for week 13. The left panels show pretreatment tissues (0 weeks) harboring proliferating parasites (amastigotes nest in red) and also (after magnification of a specific area) dye-retaining dormant amastigotes (blue). 2D orthoslicing of this area depicts both parasite populations. Comparable imaging results were achieved in 3 independent animals.

Data S1: Raw data pertaining to this manuscript.