

1 Archaeosomes display immunoadjuvant potential for a vaccine against

2 Chagas disease

3 Leticia H. Higa <sup>a</sup>; Ricardo S. Corral <sup>b</sup>; María José Morilla <sup>a</sup>; Eder L. Romero <sup>a</sup>; Patricia B. Petray <sup>b,\*</sup>

4  
5 <sup>a</sup> Programa de Nanomedicinas, Departamento de Ciencia y Tecnología, Universidad Nacional de  
6 Quilmes, Buenos Aires, Argentina; <sup>b</sup> Servicio de Parasitología y Enfermedad de Chagas, Hospital  
7 de Niños Dr. R. Gutiérrez, Buenos Aires, Argentina

8  
9 \*Correspondence to: Patricia B. Petray, E-mail: ppetray@conicet.gov.ar

10  
11 Key words: archaeosomes, *Trypanosoma cruzi*, vaccine adjuvants, nanotechnology, immunology

12  
13 Abbreviations

14 ARC, archaeosomes; ARC-TcAg, *Trypanosoma cruzi* antigens entrapped in ARC; TcAg, *T. cruzi*  
15 antigens; TPL, total polar lipids; BSA, bovine serum albumin; PBS, phosphate buffered saline;  
16 ELISA, enzyme-linked immunosorbent assay.

17  
18 Running title

19 Chagas disease protection mediated by *T. cruzi* antigens-loaded archaeosomes

20  
21 Disclosure of potential conflicts of interest

22 No potential conflicts of interest were disclosed

23 ABSTRACT

24 Archaeosomes (ARC), vesicles made from lipids extracted from Archaea, display strong adjuvant  
25 properties. In this study, we evaluated the ability of the highly stable ARC formulated from total  
26 polar lipids of a new *Halorubrum tebenquichense* strain found in Argentinean Patagonia, to act as  
27 adjuvant for soluble parasite antigens in developing prophylactic vaccine against the intracellular  
28 protozoan *T. cruzi*, the etiologic agent of Chagas disease. We demonstrated for the first time that  
29 C3H/HeN mice subcutaneously immunized with trypanosomal antigens entrapped in these ARC  
30 (ARC-TcAg) rapidly developed higher levels of circulating *T. cruzi* antibodies than those measured  
31 in the sera from animals receiving the antigen alone. Enhanced humoral responses elicited by ARC-  
32 TcAg presented a dominant IgG2a antibody isotype, usually associated with Th1-type immunity  
33 and resistance against *T. cruzi*. More importantly, ARC-TcAg-vaccinated mice displayed reduced  
34 parasitemia during early infection and were protected against an otherwise lethal challenge with the  
35 virulent Tulahuén strain of the parasite. Our findings suggest that, as an adjuvant, *H.*  
36 *tebenquichense*-derived ARC may hold great potential to develop a safe and helpful vaccine against  
37 this relevant human pathogen.

38

39 Chagas disease or American trypanosomiasis is a neglected tropical disease caused by the  
40 protozoan parasite *Trypanosoma cruzi* and has a widespread distribution in Latin America. WHO  
41 estimate that near 15 million individuals are infected worldwide and 50,000 children and adults die  
42 annually as a result of clinical complications of *T. cruzi*-induced heart disease and their lack of  
43 effective treatment.<sup>1</sup> The risk of transmission of the disease is high because the infection has been  
44 detected in non-endemic areas of the Americas and Europe due to large scale migrations. In light of  
45 these problems, it is essential to develop new strategies for the prevention and control of Chagas  
46 disease. At present, vaccines and immunotherapies targeted at *T. cruzi* infection are practically non-

47 existent. In parallel with the efforts toward the identification of vaccine candidates, several  
48 adjuvants have been assayed to generate protective immunity to *T. cruzi*, but with limited success.  
49 <sup>2,3</sup> In recent years, an increasing body of evidence has revealed the strong adjuvant properties of  
50 ARC.<sup>4-6</sup> These vesicles enclosed by one or more bilayers prepared with total polar lipids (TPL)  
51 extracted from microorganisms belonging to the domain Archaea are more avidly internalized, both  
52 *in vitro* and *in vivo*, by macrophages and antigen presenting cells than conventional liposomes.<sup>7,8</sup>  
53 They also differ from liposomes in that the inclusion of immunomodulators is not necessary to  
54 improve the adjuvancy beyond that of a simple depot effect,<sup>9</sup> favoring scale up production.

55 In this regard, in an earlier study we reported the ability of ARC composed of the TPL of a  
56 new *H. tebenquichense* strain found in Argentinean Patagonia to elicit potent antibody responses to  
57 entrapped bovine serum albumin (BSA) in mice.<sup>10</sup>

58 ARC have demonstrated great potential as adjuvant for immunogens aimed at killing  
59 intracytoplasmic bacterial pathogens such as *Listeria monocytogenes*.<sup>11</sup> However, the ability of  
60 ARC-based vaccines to protect against intracellular protozoan parasites has yet to be tested.

61 The goal of our current study was to evaluate whether *H. tebenquichense*-derived ARC may  
62 serve as adjuvant for soluble parasite antigens in developing prophylactic *T. cruzi* vaccine.

63 *T. cruzi* protein antigens (TcAg) present in a whole homogenate (WH) of parasites were  
64 prepared from epimastigote forms disrupted by pressure-depressure as previously described.<sup>12</sup>

65 ARC containing TcAg (ARC-TcAg) were prepared as state in Gonzalez et al.,<sup>10</sup> except that  
66 TcAg in phosphate buffered saline (PBS, 2.5 mg/ml) was used as the aqueous phase for the  
67 hydration of the thin lipidic film. Proteins were quantified by Bradford method,<sup>13</sup> and  
68 phospholipids quantified by a colorimetric method.<sup>14</sup>

69 Female 6-8-week-old C3H/HeN mice obtained from University of Buenos Aires, Argentina,  
70 were selected for *in vivo* efficacy studies. Research was conducted according to the National  
71 Research Council's guide for animal care and was approved by our internal Ethics Committee.  
72 Groups of five mice were immunized subcutaneously (sc) in the back on days 0, 14 and 21 with  
73 12.5 µg of free TcAg in PBS or 12.5 µg of ARC-TcAg. Control mice were injected with equivalent  
74 amount of empty ARC. The injection volume was 50 µl.

75 To evaluate humoral response, blood was collected from the tail vein at 21 days after the last  
76 immunization and sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for the  
77 presence of anti-*T. cruzi* antibodies as previously described.<sup>15</sup> Briefly, the antigen added to the  
78 plates was *T. cruzi* proteins present in a WH of parasites (200 µg/ml). The secondary antibody  
79 conjugated with peroxidase was goat anti-mouse IgG (1:5000, Pierce, Rockford, IL, Catalog #  
80 0031430) and the substrate was 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid ) (ABTS,  
81 Sigma-Aldrich Co, St. Louis, MO). Each serum was analyzed in two-fold serial dilutions. The  
82 optical density (OD) was measured at 405 nm using an ELISA reader (Multiskan Ex, Thermo  
83 Labsystems, Vantaa, Finland). End-point titers were defined as the highest serum dilution that  
84 resulted in an OD value greater than that of the mean + three standard deviations of preimmune  
85 mouse sera.

86 Detection of IgG subclass responses was performed as described above, except that the  
87 secondary antibodies were specific for mouse IgG1 and IgG 2a (1:1000, Santa Cruz Biotechnology,  
88 Santa Cruz, CA, Catalog # sc-2060 and sc-2061 respectively).

89 Immunized animals were challenged intraperitoneally (ip) at 4 weeks postboost with 150  
90 bloodstream trypomastigotes of Tulahuén strain of *T. cruzi*. Parasitemia was monitored by daily  
91 counting of number of trypomastigotes per 5 ul of fresh blood,<sup>16</sup> and mortality was recorded.

92 Data were analyzed using GraphPadPrism 5.0 software (GraphPad Software Inc., San  
93 Diego, CA). The Student's *t* test, Mann-Whitney and Fisher's exact tests were conducted to  
94 compare the possible differences between the mean values of the different groups. *P* values of <  
95 0.05 were considered to be statistically significant.

96 The ARC preparations were multilamellar, with a mean size of  $564 \pm 22$  nm and Z potential  
97 of -50 mV. The amount of antigen (proteins) and phospholipids contained in ARC was 40  $\mu$ g/ml  
98 and 20 mg/ml, respectively. The protein/lipid ratio was 2  $\mu$ g/mg. Following sc immunization with  
99 ARC-TcAg, mice exhibited serum specific IgG antibody titers between 3 and 6-fold higher (*p*=  
100 0.007) than those observed in TcAg group (Fig. 1A). As expected, immunization with empty ARC  
101 failed to evoke any anti-*T. cruzi* IgG response. After vaccination, the analysis of IgG isotype  
102 profiles revealed that both TcAg-specific IgG1 and IgG2a antibodies were induced in the ARC-  
103 TcAg and free TcAg groups. However, the IgG2a/IgG1 ratio for ARC-TcAg group was  
104 significantly (*p*=0.04) higher than that calculated for TcAg group (2.9 vs. 0.8, respectively, Fig.  
105 1B).

106 When mice vaccinated with ARC-TcAg were challenged with bloodstream Tulahuén  
107 trypomastigotes, we observed a reduction (*p*=0.03) in bloodstream parasite levels at the peak of  
108 parasitemia (17-19 dpi) when compared with animals that received free TcAg (Fig. 2A). Also,  
109 statistical analysis revealed a significant (*p*=0.04) difference in mortality rates between both groups.  
110 While all animals vaccinated with ARC-TcAg survived lethal challenge, only 20% of TcAg  
111 immunized mice remained alive after 31 days of infection (Fig. 2B). Another group of naive mice  
112 was infected with the same number of trypomastigotes and showed 100% of mortality at the peak of  
113 parasitemia. In addition, all control mice vaccinated with empty ARC developed fatal infection  
114 within 25 days post-infection.

115

116 DISCUSSION

117 In recent years, an increasing body of evidence has revealed the strong adjuvant properties  
118 of archaeosomes prepared from different archaeobacteria.<sup>5</sup> Particularly, in an earlier study we  
119 demonstrated the adjuvant activity of archaeosomes formulated from total polar lipids of a new *H.*  
120 *tebenquichense* strain found in Argentinean Patagonia when they were sc administered along BSA  
121 in mice.<sup>10</sup>

122 We herein used a murine model of acute chagasic infection to assess the potential of these  
123 new archaeosomes to act as adjuvanting vesicles with incorporated TcAg for prophylactic  
124 vaccination against *T. cruzi*.

125 We demonstrated that vaccination with ARC-TcAg induces enhanced type-1 immunity  
126 against parasite infection as measured by *T. cruzi*-specific IgG2a response in C3H/HeN mice. In our  
127 earlier study, upon sc immunization of this mouse strain, BSA entrapped in ARC elicited similar  
128 levels of both IgG1 and IgG2a.<sup>10</sup> Thus, we foresaw a balanced antibody isotype distribution in mice  
129 immunized with ARC-TcAg. Unexpectedly, the increased level of protection observed in these  
130 vaccinated animals was reflected by a prevalence of the anti-*T. cruzi* IgG2a fraction. The reason for  
131 this discrepancy is likely due to the different nature of the immunizing antigens. Previous studies  
132 have indicated that a dominant Th1 immune response is essential for the early control of Chagas  
133 disease.<sup>17</sup> It is known that circulating antibodies play a role in parasite killing and antibody  
134 titer/specificity, or a combination of these factors, are important in resistance to *T. cruzi* infection.  
135 Moreover, an efficient protective response against *T. cruzi* requires the induction of IgG2a, a Th1-  
136 type immunity-associated isotype.<sup>18</sup> Therefore, we hypothesized that the Th1-biased response  
137 elicited by ARC-TcAg in immunized mice would help confer protection against acute chagasic  
138 infection. To demonstrate this, vaccinated mice were then challenged with one of the most virulent

139 strains of *T. cruzi*.<sup>19</sup> Vaccination with ARC-TcAg clearly limited the course of *T. cruzi* infection in  
140 mice in terms of parasitemia and mortality.

141 Our study focuses on the early humoral immunity after challenge that contributes to control  
142 acute *T. cruzi* infection. The longer-term persistence of ARC-TcAg-induced specific antibody titers  
143 is presently unknown. However, based on our previous findings, it is conceivable that the ARC-  
144 TcAg vaccine is likely to develop lasting primary IgG2a response and enhanced immunological  
145 memory.<sup>10</sup> Even though antibodies may be seen as reliable surrogate predictors of protection by  
146 vaccines, it is widely accepted that cell-mediated immune functions are critical for eradicating  
147 infections caused by intracellular pathogens, including *T. cruzi*. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets  
148 appear to be important for the generation of effective immunoprotection against this protozoan  
149 infection and it is therefore desirable that the ARC-TcAg vaccine be capable of eliciting such  
150 cellular responses. Nevertheless, the lack of experimental data to clarify the ability of ARC-TcAg to  
151 raise cell-mediated protective immunity is a shortcoming of our current study. More extensive  
152 investigations on the induction of long-term memory and cellular responses upon immunization  
153 with ARC-Tc Ag, including passive transfer of antibodies and/or immune cells, will be performed  
154 in order to elucidate the protective activity of our formulation.

155 The mechanism responsible for adjuvancy of ARC remains elusive. ARC have been  
156 characterized as poor inducers of innate immunity via toll-like or CD1 receptors.<sup>20, 21</sup> However, the  
157 presence of glyco-portions of archaetidyl phosphate groups glycosidically linked to short  
158 oligosaccharides,<sup>22, 23</sup> seems to be important to the adjuvanting process. Particularly for *H.*  
159 *tebenquichense*-derived ARC, their unique content of archaetidyl phosphatidylglycerol,  
160 phosphatidylglycerophosphate methyl ester and glycosilated sulpholipids, added to the presence of  
161 mannose-containing archaeolipids,<sup>24</sup> enabling interaction with specific receptors on APC, probably  
162 contributed to the enhanced immunogenicity of the ARC-TcAg preparation. Next steps should

163 include the exploration of *T. cruzi* vaccines constituted by more defined parasite antigens  
164 formulated in ARC.

165 Chagas disease is increasingly understood as a problem of parasite persistence within the  
166 host, rather than primarily as a result of an inappropriate immune response driving pathology,<sup>25</sup>  
167 which has generated much interest in anti-*T. cruzi* vaccine development. Nonetheless, the potential  
168 harmfulness, complexity, expensiveness and difficulties to scale up some promising vaccine  
169 approaches can spoil further attempts of industrial production and acceptance by regulatory  
170 organisms. In this regard, ARC can be produced by scalable techniques and from sustainable  
171 sources. Remarkably, these lipid vesicles are derived from LPS-free archaea and have displayed  
172 low toxicity upon parenteral administration in rodents.<sup>26</sup>

173 In conclusion, this is the first demonstration that *T. cruzi* antigens can be incorporated  
174 successfully into ARC and, upon sc inoculation in mice, the resulting immunogen is capable of  
175 priming a protective response against an intracellular parasite infection. These findings indicate that  
176 ARC show promise as safe and helpful carrier-adjuvant for the design of future vaccines against  
177 this human pathogen.

178

#### 179 Acknowledgements

180 This work was supported in part by grants from the National Research Council (CONICET,  
181 Argentina). PBP, ELR; RSC and MJM are members of the Research Career Program from  
182 CONICET. LHH is a fellow from CONICET. We thank Dr. Mónica Esteva (National Institute of  
183 Parasitology Dr. Mario Fatała Chabén, Buenos Aires, Argentina) for kindly providing the Tulahuén  
184 strain of *T. cruzi*

185

186



187 References

- 188 1. The Special Programme for Research and Training in Tropical Diseases (TDR/WHO).  
189 Reporte sobre la Enfermedad de Chagas. Buenos Aires, Argentina. Guhl F, Ladzins-Helds  
190 JK, eds, 2007: 97.
- 191 2. Garg N, Bhatia V. Current status and future prospects for a vaccine against American  
192 trypanosomiasis. *Expert Rev Vaccines* 2005; 4: 867-80.
- 193 3. Quijano-Hernández I, Dumonteil E. Advances and challenges towards a vaccine against  
194 Chagas disease. *Hum Vaccin* 2011; 7: 1184-91.
- 195 4. Sprott GD, Tolson DL, Patel GB. Archaeosomes as novel antigen delivery systems. *FEMS*  
196 *Microbiol Lett* 1997; 154: 17-22.
- 197 5. Krishnan L, Dicaire CJ, Patel GB, Sprott GD. Archaeosome vaccine adjuvants induce  
198 strong humoral, cell-mediated, and memory responses: comparison to conventional  
199 liposomes and alum. *Infect Immun* 2000; 68: 54-63.
- 200 6. Li Z, Zhang L, Sun W, Ding Q, Hou Y, Xu Y. Archaeosomes with encapsulated antigens for  
201 oral vaccine delivery. *Vaccine* 2001; 29: 5260-6.
- 202 7. Sprott GD, Sad S, Fleming LP, Dicaire CJ, Patel GB, Krishnan L. Archaeosomes varying in  
203 lipid composition differ in receptor-mediated endocytosis and differentially adjuvant  
204 immune responses to entrapped antigen. *Archaea* 2003; 1: 151-64.
- 205 8. Krishnan L, Sad S, Patel GB, Sprott GD. The potent adjuvant activity of archaeosomes  
206 correlates to the recruitment and activation of macrophages and dendritic cells in vivo. *J*  
207 *Immunol* 2001; 166: 1885-93.
- 208 9. Nordly P, Korsholm KS, Pedersen EA, Khilji TS, Franzyk H, Jorgensen L, et al.  
209 Incorporation of a synthetic mycobacterial monomycoloyl glycerol analogue stabilizes

- 210 dimethyldioctadecylammonium liposomes and potentiates their adjuvant effect in vivo. Eur  
211 J Pharm Biopharm 2011; 77: 89-98.
- 212 10. González RO, Morilla MJ, Cutrullis RA, Corral RS, Petray PB, Romero EL. Archaeosomes  
213 made of *Halorubrum tebenquichense* total polar lipids: a new source of adjuvancy. BMC  
214 Biotechnology 2009; 9: 71.
- 215 11. Conlan JW, Krishnan L, Willick GE, Patel GB, Sprott GD. Immunization of mice with  
216 lipopeptide antigens encapsulated in novel liposomes prepared from the polar lipids of  
217 various Archaeobacteria elicits rapid and prolonged specific protective immunity against  
218 infection with the facultative intracellular pathogen, *Listeria monocytogenes*. Vaccine 2001;  
219 14: 3509-17.
- 220 12. Segura EL, Vazquez C, Bronzina A, Campos JM, Cerisola JA, Gonzalez Cappa SM.  
221 Antigens of the subcellular fraction of *Trypanosoma cruzi*. Part II: Flagellar and membrane  
222 fraction. J Protozool 1997; 24: 540-3.
- 223 13. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of  
224 protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.
- 225 14. Stewart JCM. Colorimetric determination of phospholipids with ammonium  
226 ferrothiocyanate. Anal Biochem 1959, 104: 10-4.
- 227 15. Corral RS, Petray PB. CpG DNA as a Th1-promoting adjuvant in immunization against  
228 *Trypanosoma cruzi*. Vaccine 2001; 19: 234-42.
- 229 16. Pizzi T. Prensa Médica Universitaria. Santiago de Chile, 1957, 38.
- 230 17. Hoft DF, Eickhoff CS. Type 1 immunity provides optimal protection against both mucosal  
231 and systemic *Trypanosoma cruzi* challenges. Infect Immun 2000; 70: 6715-25.

- 232 18. Powell MR, Wassom DL. Host genetics and resistance to acute *Trypanosoma cruzi* infection  
233 in mice. I. Antibody isotype profiles. *Parasite Immunol* 1993; 15: 215-21.
- 234 19. Taliaferro WH, Pizzi T. Connective tissue reactions in normal and immunized mice to a  
235 reticulotropic strain of *Trypanosoma cruzi*. *J Infect Dis* 1995; 96: 199-226.
- 236 20. Krishnan L, Gurnani K, Dicaire CJ, van Faassen H, Zafer A, Kirschning CJ, et al. Rapid  
237 clonal expansion and prolonged maintenance of memory CD8<sup>+</sup> T cells of the effector  
238 (CD44<sup>high</sup>CD62L<sup>low</sup>) and central (CD44<sup>high</sup>CD62L<sup>high</sup>) phenotype by an archaeosome  
239 adjuvant independent of TLR2. *J Immunol* 2007; 178 : 2396–2406.
- 240 21. de la Salle H, Mariotti S, Angenieux C, Gilleron M, Garcia-Alles L-F, Malm D, et al.  
241 Assistance of microbial glycolipid antigen processing by CD1e. *Science*, 2005; 310: 1321–  
242 24.
- 243 22. Krishnan L, Sad S, Patel GB, Sprott GD. Archaeosomes induce long-term CD8<sup>+</sup> cytotoxic T  
244 cell response to entrapped soluble protein by the exogenous cytosolic pathway, in the  
245 absence of CD4<sup>+</sup> T cell help1. *J Immunol* 2000; 165: 5177–85.
- 246 23. Sprott GD, Dicaire CJ, Côté J-P, Whitfield DM. Adjuvant potential of archaeal synthetic  
247 glycolipid mimetics critically depends on the glyco head group structure. *Glycobiology*  
248 2008; 18: 559–65.
- 249 24. Higa LH, Schilrreff P, Perez AP, Iriarte MA, Roncaglia DI, Morilla MJ, et al.  
250 Ultradeformable archaeosomes as new topical adjuvants. *Nanomedicine* 2012, in press.
- 251 25. Tarleton RL. Chagas disease: a role for autoimmunity? *Trends Parasitol.*, 2003; 19: 447-51.
- 252 26. Esko JD, Doering TL, Raetz CRH. Eubacteria and Archaea. In: Varki A, Cummings RD,  
253 Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME, eds. *Essentials of*

254 Glycobiology. 2nd edition. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press,  
255 2009, Chapter 20.

256

257

258

259

260

261

262

263

264

265

266

267

268

269

270

271

272

273

274

275

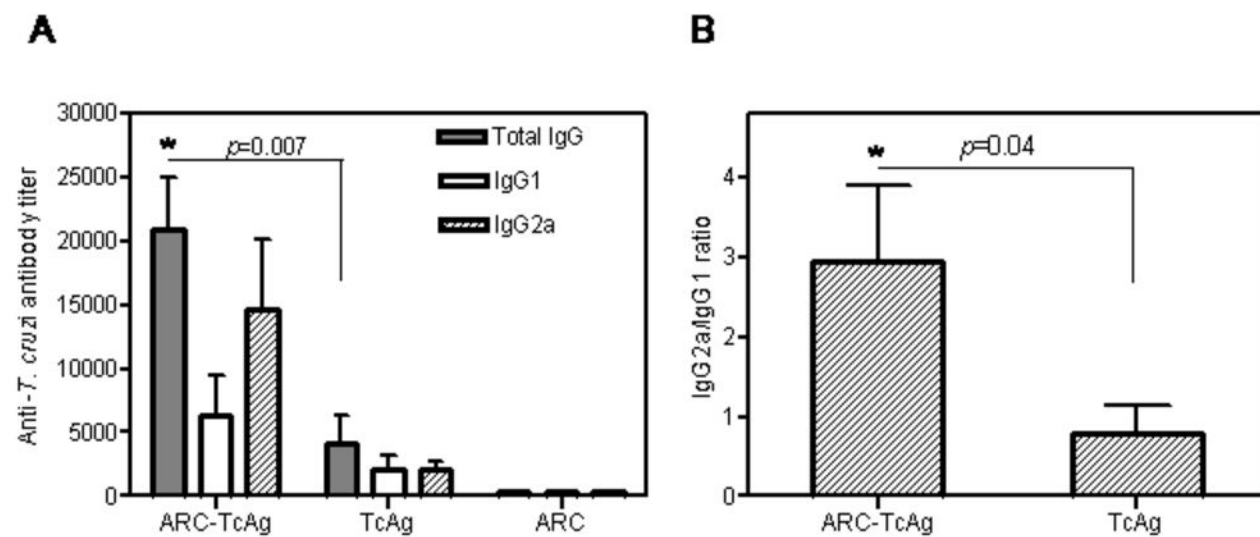
276 Legends

277 Fig. 1. Induction of humoral response to *T. cruzi* in vaccinated C3H/HeN mice. (A) ELISA analysis  
278 of antibody isotypes 3 weeks after the last immunization. (B) Ratio of IgG2a to IgG1 antibody  
279 titers. Data represent mean  $\pm$  SEM of two independent experiments.

280

281 Fig. 2. The effect of vaccination on the parasitemia (A) and mortality (B) of C3H/HeN mice  
282 infected with *T. cruzi*. \*  $p=0.03$ ; #  $p=0.04$ . Results are representative of two independent  
283 experiments.

**Figure 1**



**Figure 2**

