Archaeosomes display immunoadjuvant potential for a vaccine against Chagas disease

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Abbreviations

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

Running title

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

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No potential conflicts of interest were disclosed

ABSTRACT

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

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

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

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

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

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

T. cruzi protein antigens (TcAg) present in a whole homogenate (WH) of parasites were prepared from epimastigote forms disrupted by pressure-depressure as previously described .[12]

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

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

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

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

Immunized animals were challenged intraperitoneally (ip) at 4 weeks postboost with 150 bloodstream trypomastigotes of Tulahuén strain of *T. cruzi*. Parasitemia was monitored by daily counting of number of trypomastigotes per 5 ul of fresh blood, [16] 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 ± 22 nm and Z potential
97 of -50 mV. The amount of antigen (proteins) and phospholipids contained in ARC was 40 μg/ml
98 and 20 mg/ml, respectively. The protein/lipid ratio was 2 μg/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

DISCUSSION

In recents years, an increasing body of evidence has revealed the strong adjuvant properties of archaeosomes prepared from different archaeobacteria. [5] Particularly, in an earlier study we demonstrated the adjuvant activity of archaeosomes formulated from total polar lipids of a new *H. tebenquichense* strain found in Argentinean Patagonia when they were sc administered along BSA in mice.[10]

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

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

139 strains of *T. cruzi*. [19] 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. [10] 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 $CD4^+$ and $CD8^+$ 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. [20, 21] However, the
157 presence of glyco-portions of archaetydil phosphate groups glycosidically linked to short
158 oligosaccharides, [22, 23] 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, [24] enabling interaction with specific receptors on APC, probably
162 contributed to the enhanced immunogenicity of the ARC-TcAg preparation. Next steps should

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

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

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

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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

dimethyldioctadecylammonium liposomes and potentiates their adjuvant effect in vivo. Eur J Pharm Biopharm 2011; 77: 89-98.

10. González RO, Morilla MJ, Cutrullis RA, Corral RS, Petray PB, Romero EL. Archaeosomes made of *Halorubrum tebenquichense* total polar lipids: a new source of adjuvancy. BMC Biotechnology 2009; 9: 71.

11. Conlan JW, Krishnan L, Willick GE, Patel GB, Sprott GD. Immunization of mice with lipopeptide antigens encapsulated in novel liposomes prepared from the polar lipids of various Archaeobacteria elicits rapid and prolonged specific protective immunity against infection with the facultative intracellular pathogen, *Listeria monocytogenes.*Vaccine 2001; 14: 3509-17.

12. Segura EL, Vazquez C, Bronzina A, Campos JM, Cerisola JA, Gonzalez Cappa SM. Antigens of the subcellular fraction of *Trypanosoma cruzi*. Part II: Flagellar and membrane fraction. J Protozool 1997; 24: 540-3.

13. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248-54.

14. Stewart JCM. Colorimetric determination of phospholipids with ammonium ferrothiocyanate. Anal Biochem 1959, 104: 10-4.

15. Corral RS, Petray PB. CpG DNA as a Th1-promoting adjuvant in immunization against *Trypanosoma cruzi*. Vaccine 2001; 19: 234-42.

16. Pizzi T. Prensa Médica Universitaria. Santiago de Chile, 1957, 38.

17. Hoft DF, Eickhoff CS. Type 1 immunity provides optimal protection against both mucosal 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+ T cells of the effector
238 (CD44highCD62Llow) and central (CD44highCD62Lhigh) 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+ cytotoxic T
244 cell response to entrapped soluble protein by the exogenous cytosolic pathway, in the
245 absence of CD4+ T cell help1. J Immunol 2000; 165: 5177–85.

246 23. Sprott GD, Dicaire CJ, Côtè 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

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

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 ± 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

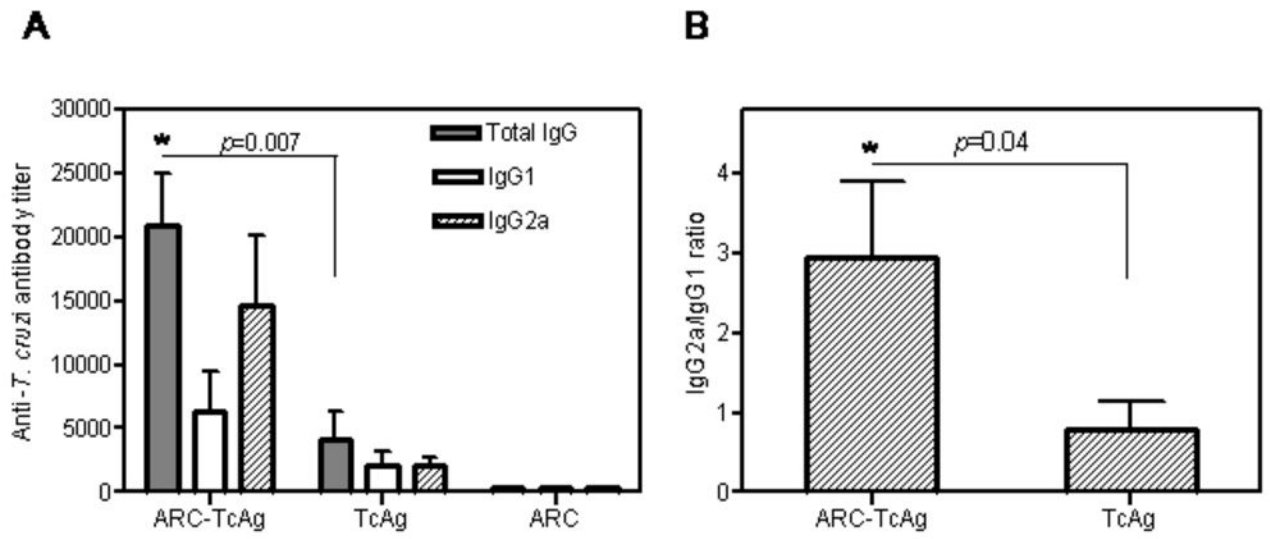
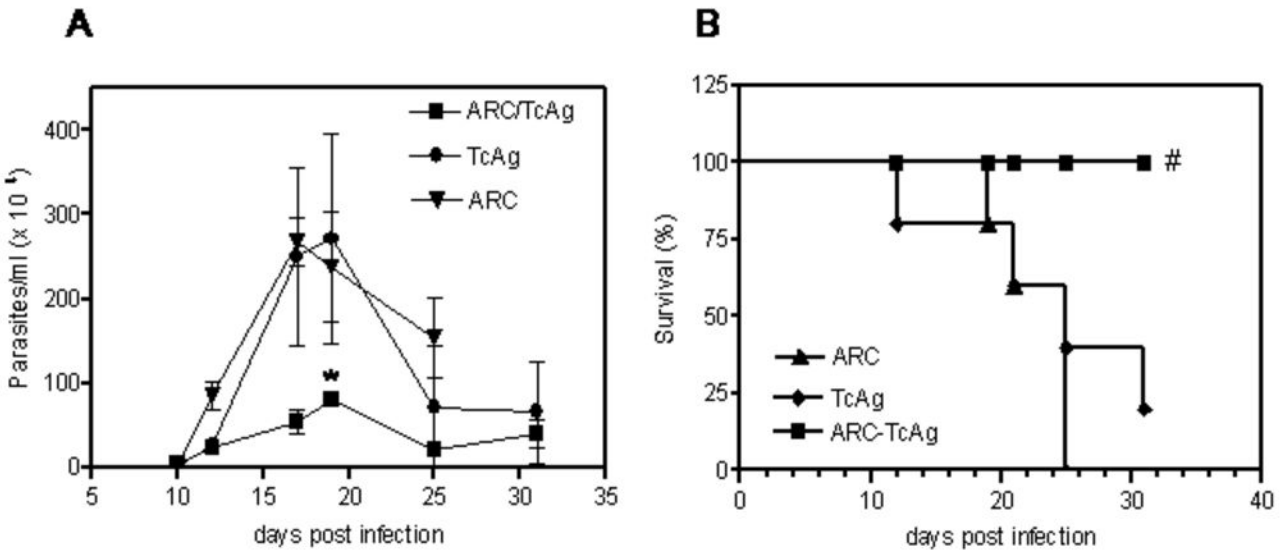

A
B
30000
25000
20000
15000
10000
5000
0
Anti-T. cruzi antibody titer
*
p=0.007
Total IgG
IgG1
IgG2a
ARC-TcAg
TcAg
ARC
4
3
2
1
0
IgG2a/IgG1 ratio
*
p=0.04
ARC-TcAg
TcAg

Figure 2


A
Parasites/ml (x 10 ⁴)
400
300
200
100
0
5
10
15
20
25
30
35
days post infection
ARC/TcAg
TcAg
ARC
*
B
Survival (%)
125
100
75
50
25
0
0
10
20
30
40
days post infection
ARC
TcAg
ARC-TcAg
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