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1 # Antigen B from *Echinococcus granulosus* enters mammalian cells by
2 # endocytic pathways

3

4 **Short title: Antigen B uptake by mammalian cells**

5

6 Edileuza Danieli da Silva[1,2], Martin Cancela[1,2], Karina Mariante Monteiro[1,3], Henrique
7 Bunselmeyer Ferreira[1,3], Arnaldo Zaha[1,2,*]
8 [1]Programa de Pós-Graduação em Biologia Celular e Molecular, Centro de Biotecnologia,
9 Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.
10 [2]Laboratório de Biologia Molecular de Cestódeos, Centro de Biotecnologia, Universidade
11 Federal do Rio Grande do Sul, Porto Alegre, Brazil.
12 [3]Laboratório de Genômica Estrutural e Funcional, Centro de Biotecnologia, Universidade
13 Federal do Rio Grande do Sul, Porto Alegre, Brazil.

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15 *Corresponding author
16 E-mail: zaha@cbiot.ufrgs.br (AZ)

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19 ## Abstract

20 Cystic hydatid disease is a zoonosis caused by the larval stage (hydatid cyst) of
21 *Echinococcus granulosus* (Cestoda, Taeniidae). The hydatid cyst develops in the viscera
22 of intermediate host as a unilocular structure filled by the hydatid fluid, which contains
23 parasitic excretory/secretory products. Antigen B (AgB) is the major component of *E.*
24 *granulosus* metacestode hydatid fluid. Functionally, AgB has been implicated in
25 immunomodulation and lipid transport. However, the mechanisms underlying AgB
26 functions are not completely known. In this study, we investigated AgB interactions with
27 different mammalian cell types and the pathways involved in its internalization. AgB uptake
28 was observed in four different cell lines, NIH-3T3, A549, J774 and RH. Inhibition of raft-
29 mediated endocytosis causes about 50 and 69% decrease in AgB internalization by RH
30 and A549 cells, respectively. Interestingly, AgB colocalized with the raft endocytic marker,
31 but also showed a partial colocalization with the clathrin endocytic marker. The results
32 indicate that raft-mediated endocytosis is the main route to AgB internalization, and that a
33 clathrin-mediated entry may also occur at a lower frequency. Cellular internalization could
34 be a requirement for AgB functions as a lipid carrier and/or immunomodulatory molecule,
35 contributing to create a more permissive microenvironment to metacestode development
36 and survival.

37

38 ## Author summary

39 Antigen B (AgB) is an oligomeric lipoprotein highly abundant in *Echinococcus granulosus*
40 hydatid fluid. AgB has already been characterized as an immunomodulatory protein,
41 capable of inducing a permissive immune response to parasite development. Also, an
42 important role in lipid acquisition is attributed to AgB, because it has been found
43 associated to different classes of host lipids. However, the mechanisms of interaction
44 employed by AgB to perform its functions remain undetermined. In this study, we

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45 demonstrate that mammalian cells are able to internalize *E. granulosus* AgB in culture and
46 found that specific mechanisms of endocytosis are involved. Our results extend the
47 understanding of AgB biological role indicating cellular internalization as a mechanism of
48 interaction, which in turn, may represent a target to intervention.
49

50 ## Introduction

51 Cystic hydatid disease (CHD), caused by the larval stage (hydatid cyst or
52 metacestode) of parasites belonging to the *Echinococcus granulosus sensu lato* (s.l.)
53 complex, is a zoonosis of worldwide occurrence, with a considerable medical and
54 economic impact [1]. CHD is endemic or hyperendemic in South America, especially in
55 Argentina, Southern Brazil, Uruguay, Chile and mountainous regions of Peru and Bolivia
56 [2]. In 2010, the World Health Organization added CHD to its list of Neglected Tropical
57 Diseases (http://www.who.int/neglected_diseases/diseases/en/). *Echinococcus granulosus*
58 *sensu stricto*, or simply *Echinococcus granulosus*, is one of the cryptic species of the *E.*
59 *granulosus* s.l. complex and is the species most widely distributed worldwide. Also, *E.*
60 *granulosus* is responsible for most cases of human CHD infections [3].

61 The adult tapeworm lives in the small intestine of a definitive canid host, and the
62 larval stage develops in the viscera of a wide range of mammal species, including
63 humans. *E. granulosus* life cycle is predominantly domestic, where dogs are the definitive
64 hosts and ungulates are the intermediate hosts [4]. The metacestode is a fluid-filled,
65 unilocular cyst containing protoescoleces in its lumen. Protoescoleces are the pre-adults,
66 infective to the definitive host, which remain quiescent and immersed in the hydatid fluid
67 (HF), which is a complex mixture of molecules of both host and parasite origin. The
68 excretory/secretory products of the metacestode are of special relevance for the host-
69 parasite relationship, as they have a greater potential to interact with host proteins and
70 cells.

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71 Antigen B (AgB) is the most abundant and the major immunodominant protein
72 among the excretory/secretory metacestode products in the HF. AgB belongs to the group
73 of hydrophobic ligand binding proteins (HLBPs), a cestode protein family whose members
74 are known by their high abundance and immunogenicity, and by their oligomeric structure,
75 comprising 7-10 kDa α-helix rich subunits [5,6]. The AgB oligomeric structure comprises 8
76 kDa subunits (AgB8/1 to AgB8/5) encoded by a multigene family [7], which are
77 differentially expressed among the parasite life-cycle stages, metacestode tissues and
78 individuals [8–10]. AgB oligomers have been observed predominantly in the molecular
79 mass range of 150-200 kDa, but aggregates with higher molecular masses have also been
80 detected [9,11].

81 It has been demonstrated that delipidated AgB is able to bind hydrophobic
82 compounds *in vitro* [12]. The lipid moiety associated with AgB was analyzed and different
83 lipids were identified, with cholesterol, phospholipids and triacylglycerides being the most
84 prominent [13]. Moreover, delipidated recombinant AgB8/2 and AgB8/3 subunits were
85 capable of transferring fatty acids analogues to artificial phospholipid membranes [14]. *E.*
86 *granulosus* genome lacks sequences for several key enzymes for fatty acid and
87 cholesterol synthesis, thus the parasite is incapable of synthesizing these compounds *de*
88 *novo* [15,16]. Hydatid cyst viability relies on the sequestration and utilization of host lipids,
89 and AgB might be involved in lipid uptake from host tissue and its transport to the parasite,
90 by stabilizing insoluble lipids into a lipoproteic particle [13].

91 In addition, AgB roles in the modulation of both innate and adaptive immunity have
92 been proposed. It has been described that neutrophils have both the recruitment inhibited
93 and hydrogen peroxide production decreased by AgB [17,18]. Besides, AgB polarizes the
94 immunological response to a Th2 profile, which is protective to the parasite [19,20].

95 Considering the two main roles attributed to AgB, immunomodulation and lipid
96 transport, it is reasonable to consider that a direct interaction with host cells and tissues

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97 should occur. In fact, it was recently demonstrated that AgB binds to macrophages and the
98 plasma membrane of inflammatory monocytes, inducing a non-inflammatory phenotype in
99 macrophages [21]. However, little is known about the molecular details of AgB-cell
100 interaction and whether AgB interacts with non-immune cells, or even enters into the cell.
101 In the present work, we investigated the ability of HF-purified AgB oligomers to
102 enter into different mammalian cell types *in vitro*, and the mechanisms involved in AgB
103 internalization. Immunopurified AgB was incubated with four distinct cell lines
104 representative of different cell types, namely hepatocytes, fibroblasts, macrophages, and
105 lung epithelial cells. We demonstrated the entry of AgB into the cytoplasm of all studied
106 cell lines. Moreover, we provided evidence that the endocytic pathways are involved in
107 AgB internalization by cells, with raft-mediated endocytosis being the prevailing one.
108

109 Methods

110 Biological material

111 Bovine viscera containing hydatid cysts from *E. granulosus* were obtained from a
112 local slaughterhouse (São Leopoldo, Brazil). Animal slaughtering was conducted
113 according to Brazilian laws and under supervision of the *Serviço de Inspeção Federal*
114 (Brazilian Sanitary Authority) of the Brazilian *Ministério da Agricultura, Pecuária e*
115 *Abastecimento*. HF was removed by punction and aspiration from individual fertile cysts
116 and kept at -80°C until use. Parasite genotyping was performed for species determination
117 [22].

118 Immunoblot

119 Aliquots of 100 µl of HF samples were resolved on SDS-PAGE 12% and
120 electrophoretically transferred onto a nitrocellulose membrane. A pool of rabbit polyclonal
121 antibodies raised against each recombinant AgB subunit (AgB8/1 to 5) were used at
122 1:70.000 dilution as primary antibody. A horseradish peroxidase-conjugated goat anti-

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123 rabbit IgG (GE Healthcare) diluted at 1:7.000 was used as the secondary antibody. Blots
124 were developed using the chemiluminescent reagent ECL Plus (Pierce, ThermoScientific)
125 and imaged in VersaDoc system (BioRad). HF samples with higher AgB content were
126 used for the protein purification step (S1A Fig).

127 ### *E. granulosus* AgB purification

128 AgB purification was carried out following the protocol described by Oriol *et al.* [23],
129 with some modifications. Briefly, parasite proteins from *E. granulosus* HF were precipitated
130 by sodium acetate (5 mM, pH 5.0) and the resultant material was resuspended in
131 phosphate-buffered saline (PBS) containing 20 μM 3,5-di-tert-butyl–4-hydroxytoluene
132 (BHT). The HF parasite enriched fraction was subjected to immunoaffinity chromatography
133 using rabbit polyclonal antibodies against the recombinant forms of AgB8/1, AgB8/2 and
134 AgB8/4. Antibodies were separately coupled to cyanogen bromide-activated Sepharose™
135 4B resin (GE Healthcare) and the previously prepared HF material was passed through
136 the columns. Bound AgB from each column was eluted with 100 mM tris-glycine pH 2.5,
137 then pooled together, dialyzed against PBS/BHT and concentrated on Amicon Ultra-15
138 centrifugal filter device, MWCO 3 kDa (Millipore). Purified AgB was analyzed on SDS-
139 PAGE 12% (S1B Fig). AgB concentration was determined using a Qubit quantitation
140 fluorometer and Quant-iT reagents (Life Technologies).

141 ### Cell cultures

142 NIH-3T3 (mouse fibroblasts), A549 (human lung adenocarcinoma), J774 (mouse
143 macrophages) and RH (rat hepatoma) cells were cultivated in DMEM containing 10% fetal
144 bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 humidified
145 environment at 37°C. J774 culture media was also supplemented with MEM non-essential
146 amino acid solution, 2 mM glutamine, 10 mM HEPES and 1 mM sodium pyruvate. All cells
147 lines were free from mycoplasma contamination.

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149 **AgB internalization assays**

150 NIH-3T3, A549, J774 and RH cells were grown on sterile glass coverslips in 35 mm
151 Petri dishes. Cell media was changed to serum-free medium and the cells were then
152 incubated with 40 μg/ml of AgB oligomers for 4 h at 37°C, or 4°C. Controls were incubated
153 with equal volume of PBS/BHT. Unbound protein was then removed by three washes with
154 cold PBS and cells were fixed in 4% paraformaldehyde/PBS at room temperature for 15
155 min.

156 In all microscopy preparations, a pool with the same proportion of polyclonal
157 antibodies against AgB8/1, AgB8/2 and AgB8/4 subunits was used as primary antibody for
158 detection of AgB oligomers. Fixed cells were permeabilized with 0.2% Triton X-100/PBS
159 and unspecific sites were blocked with 5% BSA in PBS-T (PBS with 0.05% Tween-20).
160 After, cells were incubated overnight at 4°C with the primary antibodies (1:500) and then
161 for 1 h with 1:200 diluted Alexa Fluor® 488-conjugated anti-rabbit secondary antibody
162 (Molecular Probes) at room temperature. Nuclei were stained with 100 nM 4',6-diamidino-
163 2-phenylindole (DAPI) (Molecular Probes). Actin was stained with 50 nM Alexa Fluor® 594-
164 conjugated phalloidin (Molecular Probes). Cells were imaged using a LSM 710 Zeiss
165 confocal microscope.

166 The fluorophore CM-DiI (Molecular Probes) was used to directly label AgB
167 oligomers, because it has affinity to the lipidic compounds associated to the protein. DiI-
168 labelled AgB was used to analyze internalization without cells fixation. AgB was labelled
169 with 5 μM CM-DiI (Molecular Probes) for 1 h at room temperature. Dye excess was
170 washing out with 5-fold the original PBS volume on Amicon Ultra-0.5 centrifugal filter
171 devices, NMWL 100 kDa (Millipore). RH cells were incubated with 40 μg/ml of DiI-labelled
172 AgB oligomers for 4 h at 37°C, washed three times with cold PBS, and immediately
173 analyzed using an Olympus FluoView 1000 confocal microscope.

174

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175 **Endocytosis inhibition assays**

176 RH and A549 cell monolayers were grown on sterile glass coverslips in six-well
177 tissue culture plates. After changing the cell media to serum-free DMEM, cells were pre-
178 treated with endocytosis inhibitors for 30 min at 37°C. A pilot test, where cells were
179 incubated with different concentrations of the inhibitors, was conducted to determine the
180 best concentration to be used. The highest concentration where >80% of the cells
181 remained attached and with little morphological alterations was chosen.

182 Genistein (Santa Cruz Biotechnology) was used at 100 µg/ml concentration and
183 chlorpormazine (Santa Cruz Biotechnology) at 5 µg/ml. AgB was then added at 40 µg/ml
184 and after incubation at 37°C for 1.5 h, the unbound proteins were removed by acidic
185 stripping (0.5 M NaCl, 0.5% acetic acid, pH 3.0) and three washes with cold PBS. Cells
186 were fixed and prepared for microscopy as described above. Cells were imaged using an
187 Olympus FluoView 1000 confocal microscope. Immunofluorescence intensity normalized
188 by cell area was assessed with ImageJ software [24]. Image analysis was done on two
189 (A549) or three (RH) independent experiments, where three microscopy fields were
190 counted for each experiment (100–300 cells/experiment).

191 **Colocalization assays**

192 RH cell monolayers were grown on sterile glass coverslips in 6-well tissue culture
193 plates. Cell media was replaced to serum-free DMEM containing 40 µg/ml AgB and the
194 distribution of internalized protein was compared with that of different endocytic markers
195 following up to 1.5 h incubation at 37°C. Endogenous transferrin receptors were labeled
196 with 50 µg/ml Alexa Fluor® 633-conjugated transferrin (Tfn) (Molecular Probes), added in
197 the last 45 min. Alexa Fluor® 555-conjugated cholera toxin subunit B (Ctx-B) (Molecular
198 Probes) at 1 µg/ml concentration was added in the last 15 min of incubation. Adsorbed and
199 unbound proteins were removed by acidic stripping (0.5 M NaCl, 0.5% acetic acid, pH 3.0)
200 and three washes with cold PBS. Cells were prepared for microscopy and imaged as

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201 described for endocytosis assay. Colocalization was assessed using JaCoP plugin from
202 ImageJ software [25]. Image analysis was done for two independent experiments.

203 **MTT reduction assays**

204 A549 and RH cells were plated onto 96-well plates at a density of 10^4 cells/well.
205 AgB oligomers were added to the cell media at 2.5 – 40 µg/ml final concentrations. After
206 24 h incubation, 0.5 mg/ml of MTT solution in PBS was added to each well and incubated
207 for a further 4 h. To solubilize formazan, 100 µl of cell lysis buffer (16% SDS, 40% N,N-
208 dimethylformamide, 2% acetic acid, pH 4.7) was added to each well and the samples were
209 incubated overnight at 37°C in a humidified incubator. Absorbance values of formazan
210 were determined at 595 nm with an automatic microplate reader (Bio-Rad, model 550).
211 Analysis was done for five independent experiments.

212 **Statistics**

213 A Kolmogorov-Smirnov was applied to verify the normality of the data. Statistical
214 significance was analyzed by unpaired Student's t-test using the GraphPad Prism 6.0
215 software. Data are expressed as mean ± SEM and p values of less than 0.05 were
216 considered statistically significant.

217

218 Results

219 **AgB oligomers are internalized by mammalian cells in culture**

220 To investigate the ability of AgB oligomers to interact with and to be internalized by
221 mammalian cells, AgB from *E. granulosus* HF was immunopurified and added to the
222 culture medium of NIH-3T3, A549, RH or J774 cells. AgB internalization was evaluated
223 after 4 h of incubation at 37°C using an immunofluorescence assay. Cells were prepared
224 for confocal microscopy by labelling AgB oligomers with polyclonal antibodies against
225 AgB8/1, 2 and 4 subunits and a secondary anti-rabbit IgG conjugated to Alexa Fluor® 488.
226 AgB signals were detected in the four cell lines tested, suggesting that AgB is able to

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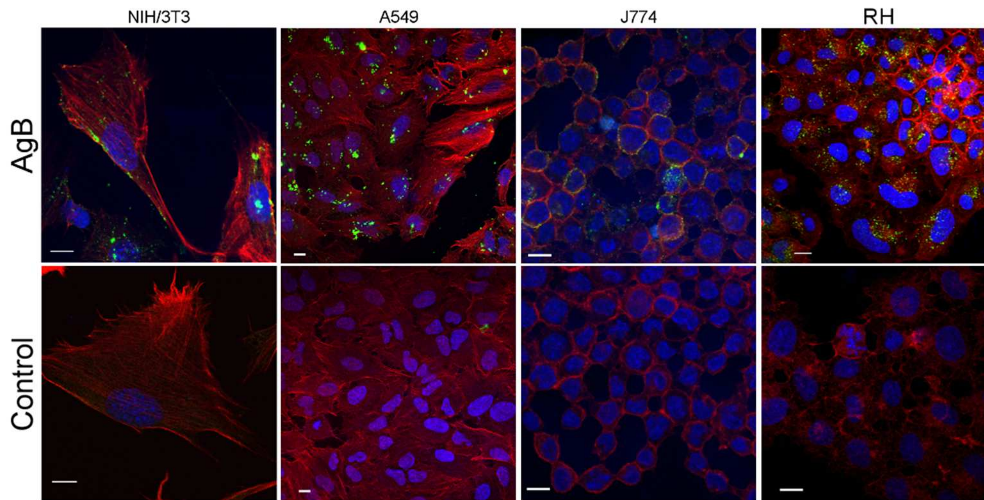
227 interact with mammalian cells by a mechanism independent of cellular type. No signals
228 were detected in the cells without AgB (Fig 1).



229

230 **Fig. 1. *E. granulosus* AgB uptake by mammalian cells in culture.** Immunofluorescence
231 assay was performed on NIH-3T3, A549, J774 and RH cells exposed to 40 µg/ml AgB for
232 4 h, and mock treated cells (Control). AgB was labeled with antibodies against AgB8/1, 2
233 and 4 subunits and an Alexa Fluor® 488-conjugated secondary antibody (green). Nuclei
234 and cytoskeleton were stained with DAPI (blue) and Alexa Fluor® 594-conjugated
235 phalloidin (red), respectively. Images are median optical sections from z-stacks obtained
236 by confocal microscopy. Scale bar, 10 µm.

237

238 To confirm the internal localization of AgB in the cells, the oligomers were labelled
239 with DiI and incubated with RH cells in the same way as before. However, the analysis on
240 confocal microscope was conducted right after incubation had been finished, without cell
241 fixation. The intermediate sections from confocal z-stacks showed higher AgB signal than
242 top or bottom sections, confirming that AgB was inside cells and not just adsorbed to cell
243 membrane (S2 Fig).

244 AgB was detected in the cell cytoplasm, but not in the nucleus. In addition, Fig 1
245 and S2 Fig show vesicular-like distribution of AgB oligomers in the cytoplasm of the cell

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246 lines analyzed, indicating an internalization through endocytosis. Supporting this idea, AgB
247 internalization does not occur when RH cells were incubated at low temperature (S3 Fig),
248 a condition known to interfere in endocytosis-dependent cellular internalization [26].

249 **Endocytic pathways involved in AgB internalization**

250 Having established that AgB oligomers could access the cytoplasmic compartment
251 of mammalian cells, we then investigated which endocytic pathway could be responsible
252 for this AgB uptake by RH and A549 cells. These two cell lines were chosen to perform the
253 following experiments in an attempt to simulate the natural situation, as liver and lungs are
254 the primary organs infected by *E. granulosus*.

255 Genistein, a tyrosine kinase inhibitor that prevents lipid raft-mediated endocytosis,
256 was used to treat RH cells; and after 30 min AgB oligomers were added to the culture
257 media and left to incubate for another 1.5 h at 37°C. We found that internalization of AgB
258 was inhibited by ~50% in RH cells treated with genistein (Figs 2A and 2B). The same
259 inhibition assay was carried out with A549 cells and we found very similar results, where
260 AgB uptake was inhibited by ~69% (Figs 2A and 2B). The results were statistically
261 significant for both cell lines (Fig 2B).

262

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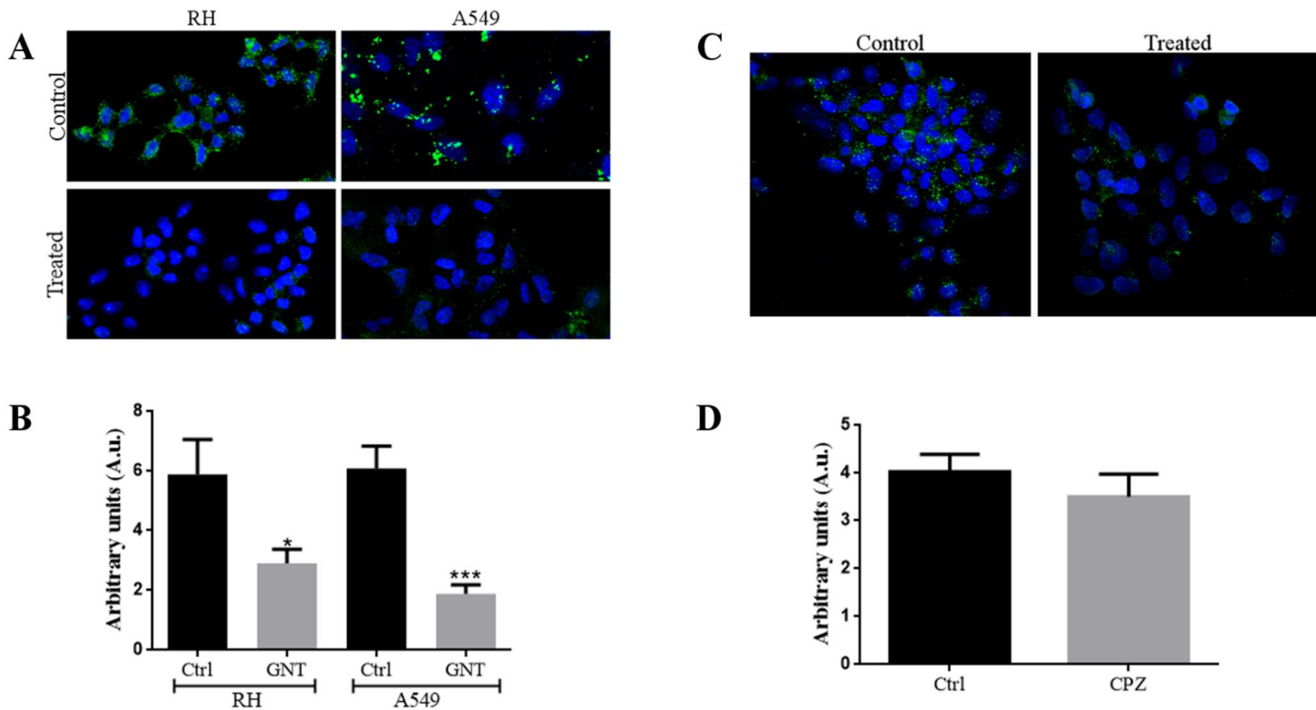

264 **Fig. 2. Raft-mediated endocytosis is the main route involved in *E. granulosus* AgB**
265 **internalization by A549 and RH cells.** *A*, inhibition of raft-mediated endocytosis by
266 genistein reduces AgB internalization. *Lower panels*, RH and A549 cells pre-treated with
267 100 µg/ml genistein, then exposed to 40 µg/ml AgB for 1.5 h. *Upper panels*, non-treated
268 cells. *B*, quantitative data for AgB internalization after genistein (GNT) treatment
269 represented in *A. C,* inhibition of clathrin-mediated endocytosis pathway by
270 chlorpromazine does not cause a significant decrease in uptake of AgB by RH cells. *Right*
271 *panel*, cells treated with 5 µg/ml chlorpromazine for 30 min, then exposed to 40 µg/ml AgB
272 for 1.5 h. *Left panel*, non-treated cells. *D*, quantitative data for AgB internalization after
273 chlorpromazine (CPZ) treatments represented in *B.* AgB was detected using antibodies
274 against AgB8/1, 2 and 4 subunits and a secondary anti-rabbit IgG Alexa Fluor® 488
275 conjugated antibody (green). Cell nuclei were labeled with DAPI (blue). Ctrl: control.
276 Measurements from three experiments with RH cells and two with A549 cells were
277 averaged. Error bars indicate SEM. *p=0.037, ***p=0.0004 according to Student's t-test.

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278 In order to test whether other endocytic pathways could be involved in AgB
279 oligomers uptake by RH cells, the inhibition assay was performed using chlorpromazine,
280 an inhibitor of clathrin-mediated endocytosis. After chlorpromazine treatment,
281 internalization of AgB was reduced by ~13%, however this was not statistically significant
282 ($p = 0.39$) (Figs 2C and 2D). The higher inhibition of AgB internalization in the genistein
283 treatment indicates that raft-mediated endocytosis is the major pathway associated with
284 AgB uptake.

285 The specificity of the inhibitors was confirmed using established endocytic markers
286 as controls. Internalization of transferrin (Tfn), which undergoes clathrin-mediated
287 endocytosis, was affected only by chlorpromazine. In contrast, genistein only reduced
288 internalization of cholera toxin subunit B (Ctx-B), which undergoes raft-mediated
289 endocytosis (S4 Fig).

290 In a complementary approach, the distribution of internalized AgB was compared
291 with that of established endocytic markers, Tfn and Ctx-B. RH cells were cultivated in the
292 presence of AgB for 1.5 h, with either Tfn or Ctx-B being added in the last 45 and 15 min
293 of incubation, respectively. Results were analyzed by confocal microscopy and the level of
294 colocalization between the two fluorophores and, consequently, the two proteins, was
295 determined according to Pearson's correlation coefficient. The results indicated that AgB
296 was colocalized with Ctx-B (Pearson's coefficient = 0.64 ± 0.02) (Fig 3), which is in
297 accordance with our previous results that raft-mediated endocytosis is involved in AgB
298 uptake. Interestingly, AgB seemed partially colocalized with Tfn (Pearson's coefficient =
299 0.49 ± 0.04), suggesting that in some degree AgB could be internalized by clathrin-
300 mediated endocytosis.

301

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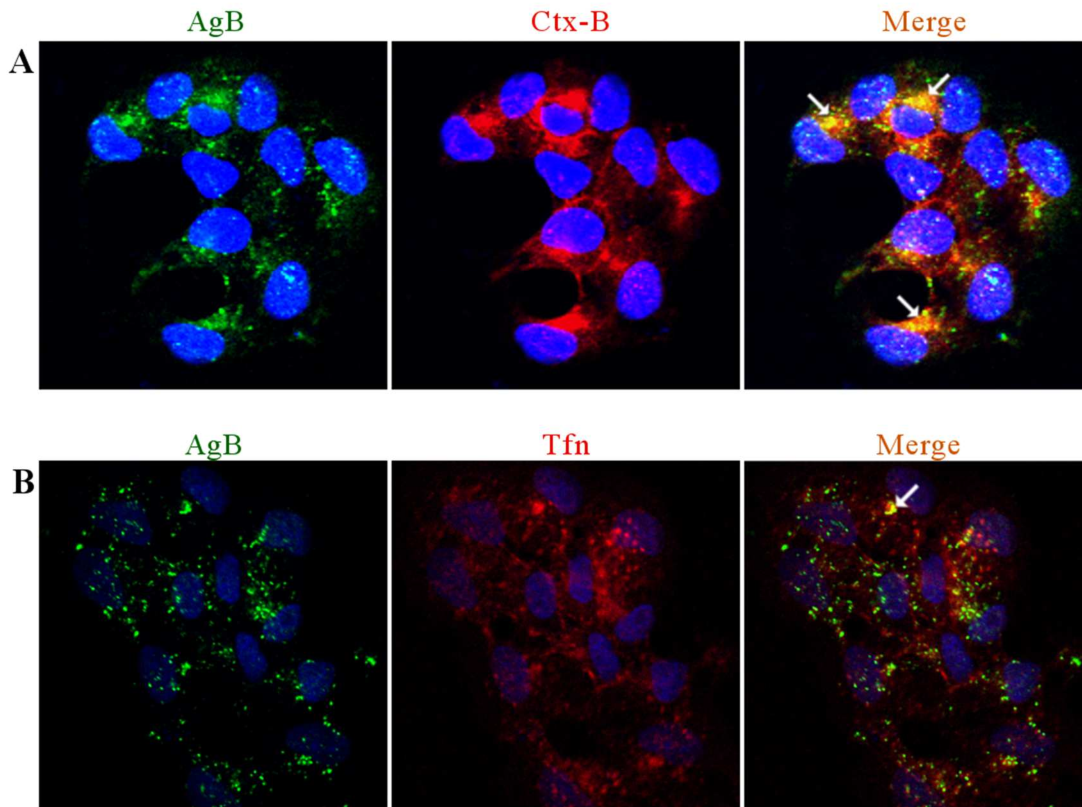

303 **Fig. 3. Internalized *E. granulosus* AgB colocalizes with protein endocytic markers in**
304 **RH cells.** Alexa Fluor® 633-conjugated Tfn was used as a marker of clathrin-mediated
305 endocytosis and Alexa Fluor® 555-conjugated Ctx-B was used as a marker of raft-
306 mediated endocytosis. Confocal microscopy images of RH cells incubated with 40 µg/ml
307 AgB and 1 µg/ml Ctx-B (A) or 50 µg/ml Tfn (B) are presented. AgB was detected using
308 antibodies against AgB8/1, 2 and 4 subunits and a secondary anti-rabbit IgG Alexa Fluor®
309 488 conjugated antibody (green). Arrows indicate colocalization points. Cell nuclei were
310 labeled with DAPI (blue). The endocytic markers are shown in red.

311

312 Altogether, the above findings provide evidence that AgB entry into mammalian
313 cells occurs mainly via raft-mediated endocytosis, although it could also occur by clathrin-
314 mediated endocytosis in a lesser extent.

315

316

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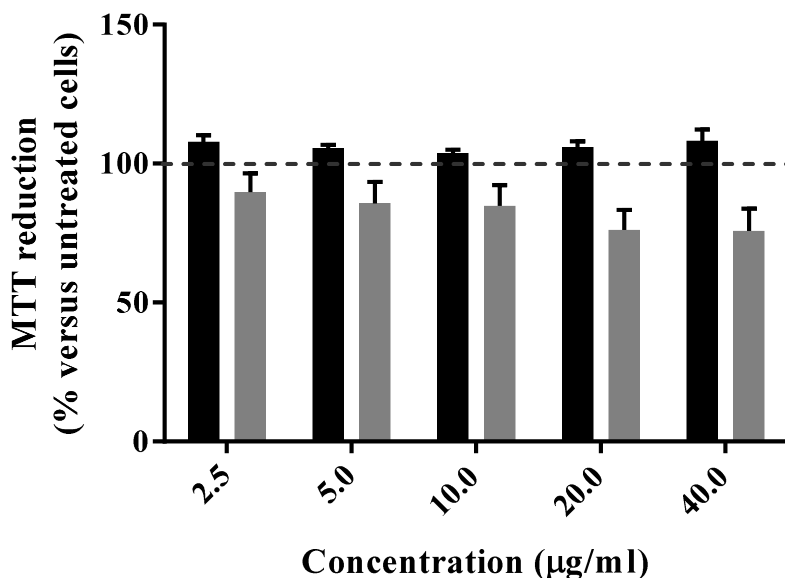
317 **AgB oligomers do not induce cellular toxicity**

318 To further investigate the possible effects of AgB oligomers internalization by
319 mammalian cells, A549 and RH cells were incubated with different concentrations of AgB
320 (2.5-40 µg/ml) for 24 h. Alterations of cell physiologic state were then evaluated by MTT
321 reduction assays. Both cell lines did not present any significant decrease in their ability to
322 metabolize MTT in presence of AgB oligomers (Fig 4). AgB oligomers do not have a
323 cytotoxic effect on cells, so this interaction is probably part of the mechanisms underlying
324 AgB function in host-parasite interplay.



325

326 **Fig. 4. MTT reduction assay.** A549 (black bars) and RH (gray bars) cells were treated for
327 24 h with the indicated concentrations of *E. granulosus* AgB oligomers. Cell viability is
328 expressed as percentage of MTT reduction measured for untreated cells and assumed as
329 100% (horizontal dashed line). Error bars correspond to the SEM values of five
330 independent experiments.

331

332 Discussion

333 A long-term growth is characteristic of the chronic infection caused by *E. granulosus*
334 metacestodes, and to allow that, different molecular mechanisms are employed by the

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335 parasite to ensure its survival and development in the host microenvironment. In an
336 attempt to evade the host's immune response and absorb nutrients from the host, the
337 parasite secretes several molecules. AgB is the major antigen in HF and has been
338 implicated in immunomodulation processes, as well as in lipid transport and uptake
339 [13,27].

340 AgB has been studied for a long time and it is known to generate a strong humoral
341 response and to modulate host immune response, which is in agreement with the idea that
342 AgB is secreted towards the outside of the hydatid cyst [27,28]. Moreover, there are many
343 studies reporting AgB effects on immune cells that support a direct AgB-cell interaction in
344 host tissue [20,21]. However, whether this interaction occurs with other cell types and what
345 underlying mechanisms are involved in this process, are still unclear. We hypothesized
346 that AgB may interact with host tissue cells surrounding the hydatid cyst to interfere with
347 host cell homeostasis, facilitating nutrient acquisition and immune evasion. In this study,
348 we demonstrated that *E. granulosus* AgB is taken up by mammalian cells *in vitro* by
349 endocytosis. Since AgB internalization seems to be independent of the cell type, it most
350 likely occurs by a ubiquitous mechanism. Thus, we further evaluate the involvement of
351 specific endocytic pathways on AgB internalization.

352 Clathrin-mediated endocytosis is the best-understood internalization pathway and
353 refers to intake of receptors and their bound ligands through vesicles which are coated by
354 the protein clathrin [29]. Among the molecules known to be internalized by this pathway is
355 the Tfn receptor, hence Tfn was used as a marker for clathrin-mediated endocytosis [30].
356 Besides the clathrin-mediated pathway, lipid rafts domains are important contributors to
357 endocytosis processes. These are heterogeneous membrane domains enriched in
358 sphingolipids and cholesterol, and are involved in the endocytosis of various receptors and
359 ligands with a multitude of mechanisms and regulation factors [31]. Ctx-B is a molecule
360 that binds to glycosphingolipid GM1 on rafts to be subsequently internalized, so we used it

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361 as a marker for raft-mediated endocytosis [32]. Chlorpromazine and genistein were used
362 in this study to inhibit clathrin- and raft-mediated endocytosis pathways, respectively.
363 Treatment with genistein was able to significantly decrease AgB oligomers uptake
364 by RH and A549 cells. Accordingly, we found that AgB colocalizes with Ctx-B in RH cells.
365 We observed a partial colocalization of AgB with Tfn and the inhibition assay with
366 chlorpromazine showed only a slight, not significant, decrease in AgB internalization. It is
367 possible that clathrin-mediated endocytosis accounts for just a small part of AgB uptake,
368 making difficult the detection of a difference after inhibition by chlorpromazine. Taken
369 together, our results are consistent with the idea that the endocytosis process is required
370 for AgB entry into mammalian cells. Indeed, raft-mediated endocytosis is most likely the
371 main pathway involved in AgB uptake by cells. However, a minor role for clathrin-mediated
372 endocytosis in AgB internalization cannot be excluded.
373 It was proposed that AgB binds to macrophages and monocytes plasma
374 membranes through a lipoprotein receptor; however no specific receptor could be
375 determined [21]. Our findings are in agreement with this idea because some lipoprotein
376 receptors, such as lectin-like oxidized LDL receptor-1 (LOX-1), LDL receptor-related
377 protein 6 (LRP6), and scavenger receptors CD36 and CD204 use raft-mediated pathways
378 for endocytosis [33–36]. Considering our results, it is also possible that more than one
379 receptor might be involved in AgB binding, so that a higher efficiency of internalization is
380 obtained. Alternatively, the receptor undergoes endocytosis by both pathways upon AgB
381 binding. This regulatory mechanism involving different endocytic routes has been
382 observed with LRP6, in which the receptor is internalized by caveolae (a raft subdomain)
383 to promote Wnt/β-catenin signaling transduction, whereas the clathrin route leads to LRP6
384 degradation [37]. AgB uptake did not induce toxicity to cells according to our MTT assay,
385 therefore internalization is more likely part of the mechanisms underlying AgB roles during
386 *E. granulosus* metacestode infection. AgB-cell interaction may be a mechanism used by

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387 the parasite to create a more permissive microenvironment for metacestode development
388 and survival. AgB presence in cytoplasm could interfere with cell metabolism, generating
389 molecules and/or signals beneficial to the parasite.

390 The lack of genes coding for several key enzymes involved in fatty acid and
391 cholesterol synthesis [15,16] reinforce the idea that AgB interaction with cells from the host
392 tissue surrounding the hydatid cyst is a suitable scenario to get lipids from biological
393 membranes or inner cell storages. A similar scenario has been described for the *Taenia*
394 *solium* metacestode, where HLBPs were able to translocate lipid analogs to parasites'
395 tissues, and also colocalize with lipid droplets in the granuloma surrounding the
396 metacestode [6]. Since lipids are essential for metacestode survival and development, the
397 understanding of molecular mechanisms employed by the parasites to acquire these host
398 macromolecules will provide potential targets for therapeutic discovery efforts.

399 AgB internalization by immune cells could influence the signalization towards anti-
400 inflammatory or alternative pathways, eliciting a host non-protective response
401 characteristic of immunoevasion processes. Similarly, in *Fasciola hepatica* a cathelicidin-
402 like protein was described to bind lipid rafts, and after internalization, to divert
403 macrophages function by suppressing lysosomal activity and, consequently, interfering
404 with antigen presentation [38].

405 The destination of AgB after entry into cells was not evaluated here, but is an
406 important issue to be addressed in further investigations in order to confirm that AgB
407 internalization is necessary for its proposed biological roles. Among many possible
408 destinations, an endocytosed protein can be routed to the late endosomes and lysosomes
409 for degradation, to the trans-Golgi network or to recycling endosomes that bring the cargo
410 back to the plasma membrane [39]. It would be interesting to know if AgB goes to the
411 recycle pathway, which would permit its further exocytosis and return to parasitic tissues,
412 or if it goes to lysosome for degradation. Further studies of colocalization with lysosomes

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413 and recycling endosomes using organelle markers described in the literature, as LAMP-1
414 and Rab-11 [40,41], should help to elucidate the fate of AgB after internalization.
415 Like AgB, cestode HLBPs are involved in parasite lipid homeostasis and
416 immunological process [6,42]. Thus, further investigations on the cellular and molecular
417 effect of HLBPs on host cells are important steps to improve the understanding of the
418 parasites biology and disease progression. Likewise, elucidating how the molecules
419 sequestered by HLBPs become available to parasites cells will help to identify potential
420 targets for the treatment and control of cestodiasis.
421

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425

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