1 ***Brucella abortus* Omp19 recombinant protein subcutaneously co-**
2 **delivered with an antigen enhances antigen-specific T helper 1 memory**
3 **responses and induces protection against parasite challenge**

4 Lorena M. Coria^a, Andrés E. Ibañez^a, Karina A. Pasquevich^a, Paula L. González
5 Cobiello^b, Fernanda M. Frank^b, Guillermo H. Giambartolomei^c and Juliana Cassataro^{a#}

6

7 Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús (IIB-
8 INTECH), Universidad Nacional de San Martín (UNSAM) –CONICET^a. Departamento de
9 Microbiología, Parasitología e Inmunología, Facultad de Medicina, UBA, Buenos Aires,
10 Argentina^b. Instituto de Inmunología, Genética y Metabolismo (INIGEM). Laboratorio de
11 Inmunogenética, Hospital de Clínicas “José de San Martín”. CONICET-UBA. Buenos Aires.
12 Argentina^c.

13

14 Running head: Omp19 is a Th1 adjuvant

15 #Address correspondence to Dr. Juliana Cassataro. e-mail:
16 jucassataro@iibintech.com.ar

17

18

19

20

21

22

23 **Abstract**

24 The discovery of effective adjuvants for many vaccines especially those with limited
25 commercial appeal, such as vaccines to poverty-related diseases, is required. In this
26 work, we demonstrated that subcutaneous co-administration of mice with the outer
27 membrane protein U-Omp19 from *Brucella* spp. plus OVA as antigen (Ag) increases
28 Ag-specific T cell proliferation and T helper (Th) 1 immune responses *in vitro* and *in*
29 *vivo*. U-Omp19 treated dendritic cells promote IFN- γ production by specific CD4⁺ T
30 cells and increases T cell proliferation. U-Omp19 co-administration induces the
31 production of Ag specific effector memory T cell populations (CD4⁺ CD44^{high}
32 CD62L^{low} T cells). Finally, subcutaneous co-administration of U-Omp19 with
33 *Trypanosoma cruzi* Ags confers protection against virulent parasite challenge, reducing
34 parasitemia and weight loss while increasing mice survival. These results indicate that
35 the bacterial protein U-Omp19 when delivered subcutaneously could be a suitable
36 component of vaccine formulations against infectious diseases requiring Th1 immune
37 responses.

38

39

40

41

42

43

44

45

46 **Introduction**

47 Changes in the nature of vaccine components and routes of administration are
48 imposing the need to develop new adjuvants. Modern adjuvant development, which in
49 spite of many hurdles is progressing, is based on enhancing and shaping vaccine-
50 induced responses without compromising safety by selectively adding well-defined
51 molecules, formulations or both. Adjuvants currently employed in human vaccines
52 licensed for use in the US and/or Europe include aluminum salts, oil-in-water emulsions
53 (MF59, AS03 and AF03), virosomes and AS04 (monophosphoryl lipid A preparation
54 (MPL) with aluminum salt) [1, 2].

55 Worth mentioning, safe and effective adjuvants are required for many vaccines
56 with limited commercial appeal, such as vaccines to infrequent (orphan) diseases or to
57 neglected and poverty-related diseases [3, 4]. So, there is also now a major interest in
58 designing vaccines capable of eliciting strong cellular immune responses of the T helper
59 1 (Th1) type [5]. The induction of such Th1 responses is for example highly desirable
60 for vaccines targeting either chronic parasite and viral diseases, infections linked to
61 intracellular pathogens or cancer (therapeutic vaccines) [6].

62 Given their potent immunostimulatory capacity, bacteria-derived molecules
63 constitute a major potential source of adjuvants. Unfortunately, whole live or killed
64 microorganisms are too toxic to be used as human adjuvants [7, 8]. We have previously
65 reported that intraperitoneal immunization with purified recombinant Omp19 from *B.*
66 *abortus* lipidated (L) or unlipidated (U) version without any adjuvant induced a Th1
67 response and also conferred protection against *Brucella* infection. Thus, our results
68 indicate that the protein region of the Omp19 lipoprotein (U-Omp19) has self-adjuvant
69 activity on cellular immune responses. Moreover, we demonstrated that U-Omp19 is
70 able to induce the maturation of murine dendritic cells (DCs) *in vivo*. Immune protective

71 responses were independent of TLR4. Also, U-Omp19 co-delivered orally induces
72 specific mucosal immune responses [9-11]. All together these results prompted us to
73 test the hypothesis that U-Omp19 is an adjuvant for co-administered Ags when
74 delivered subcutaneously. In this work we studied the systemic adjuvant capacity of the
75 protein U-Omp19 when is co-administered with a model Ag (OVA) subcutaneously and
76 also assessed its capacity to increase protection in a vaccine formulation against *T. cruzi*
77 infection in mice.

78

79

80

81

82

83

84

85

86

87

88

89

90 **Material and methods**

91 *Ethics Statement*

92 All experimental protocols with animals were conducted in strict accordance with
93 international ethical standards for animal experimentation (Helsinki Declaration and its
94 amendments, Amsterdam Protocol of welfare and animal protection and National
95 Institutes of Health, USA NIH, guidelines). The protocols of this work were also
96 approved by the Institutional Committee for the Care and Use of Laboratory Animals
97 (CICUAL) from the University of Buenos Aires and CICUAE from University of San
98 Martin.

99 *Animals*

100 Eight week old female BALB/c or C57BL/6 mice were purchased from University of
101 La Plata or from University of San Martin (UNSAM) and housed in the animal
102 resources facility of University of Buenos Aires (UBA) or UNSAM. OT-II RAG1 (OT-
103 II) and D011.10 mice were obtained from The Jackson Laboratory and were bred in the
104 animal facility of UNSAM.

105 *Aggs and adjuvants*

106 Chicken egg OVA grade V (Sigma-Aldrich) was used as model Ag. Recombinant
107 unlipidated (U)-Omp19 was obtained as previously described [9]. LPS contamination
108 from U-Omp19 was adsorbed with Sepharose-polymyxin B (Sigma). Endotoxin
109 determination was performed with Limulus amoebocyte chromogenic assay (LONZA).
110 In some experiments U-Omp19 fully digested with proteinase K was used as control. U-
111 Omp19 was treated with proteinase K-agarose from *Tricirachium album* (Sigma) as

112 described [10]. All U-Omp19 preparations used contained <0.1 endotoxin units per mg
113 of protein. Incomplete Freund Adjuvant (IFA) and Complete Freund Adjuvant (CFA)
114 were purchased from Sigma.

115 *Immunizations*

116 BALB/c mice were s.c. immunized once a week during 3 weeks with i) OVA (50µg), ii)
117 OVA (50µg) plus U-Omp19 (100µg), iii) OVA (50µg) plus CFA (100 µl) or iv) saline.
118 Three weeks after the last immunization mice were sacrificed to perform cellular
119 experiments.

120 *Determination of T helper immune responses*

121 Splenocytes from immunized mice were cultured and stimulated with OVA, or
122 complete medium alone and production of IFN- γ , IL-4, IL-10 and IL-17 were
123 determined by ELISA as described [12] .

124 *DTH test*

125 Three weeks after the last boost the delayed-type hypersensitivity (DTH) response was
126 measured by determining footpad swelling 48h after subcutaneous injection of Ag into
127 hind footpad as described [12].

128 *Proliferation assay*

129 Spleen cells from immunized mice were tested in a standard [3 H] thymidine
130 incorporation assay. Effector cells were suspended in complete medium and cultured
131 with soluble OVA (100 or 1000µg/ml) or Concanavalin A (5µg/ml) as control. Cultures
132 were incubated for 5 days at 37°C and pulsed for 18h with 1 µCi/well of [3 H]

133 thymidine. Cells were harvested and radioactivity was measured with a liquid
134 scintillation counter. The mean number of counts per minute (cpm) from triplicate wells
135 was determined. Data are presented as mean±SD from five animals per group.

136 *Flow cytometry analysis of CD4⁺ T cells*

137 Splenocytes from immunized mice were incubated with OVA (10µg/ml) plus OVA323–
138 339 peptide (KISQAVHAAHAEINEAGOVA) restricted to MHCII (1µg/ml) and
139 mitomycin-treated APCs (A20J cells stimulated with OVA for 24h) in complete
140 medium supplemented with IL-2 (10U/ml) for 5 days at 37°C and 5% CO₂. After
141 stimulation, cells were stained with anti- CD4, CD44 and CD62L. Flow cytometry
142 analysis was performed using FACSariaII flow cytometer (BD Biosciences) and further
143 analyzed using FlowJo 7.5 software (TreeStar Inc).

144 *Determination of Ab responses*

145 Serum was obtained 3 weeks after last immunization to study OVA-specific humoral
146 response (total IgG, IgG1 and IgG2a isotypes) by ELISA as described previously [10].
147 Cut-off values for the ELISA assays were calculated as the mean specific OD plus 3 SD
148 from 20 sera from non-immunized mice. Serum titers were established as the reciprocal
149 of the last dilution yielding an OD higher than the cut-off.

150 *Bone marrow derived DCs (BMDCs)*

151 DCs were generated from bone marrow (BM) mononuclear cells from wild-type
152 BALB/c or C57BL/6 mice as described [12].

153

154 *In vitro Ag-presentation assay*

155 BMDCs (CD11c⁺ MHCII^{low}) from BALB/c mice (1x10⁶) were pulsed with complete
156 medium, OVA (50µg/ml), OVA plus U-Omp19 (100µg/ml) or OVA plus LPS (1ng/ml)
157 for 24h. Then, DCs were washed and co-cultured with transgenic DO11.10 spleen cells
158 (4x10⁶ cells) at 37°C for 72h. During the last 5h cells were treated with brefeldin A.
159 Afterwards, cells were washed and stained with anti-CD4 and then fixed, permeabilized
160 with saponin and stained with anti-IFN-γ. Production of intracellular IFN-γ by CD4⁺ T
161 cells was assessed by flow cytometry. Similar experiments were performed using the
162 chimera protein (GFP-OTII) as Ag [13]. In these experiments the IFN-γ production was
163 measured in CD4⁺ T cells from OTII mice.

164 *Adoptive transfer of OT-II cells and in vivo CD4⁺ T-cell proliferation*

165 Splenocytes from OT-II mice were labeled with 5 µM CFSE (Molecular Probes) prior
166 to i.v. injection. One day before immunization, 10x10⁶ OT-II cells were injected i.v in
167 C57BL/6 sex-matched recipients. Transferred mice received a single s.c. dose of OVA
168 (30µg) plus U-Omp19 (100µg), proteinase K digested U-Omp19 (100µg), LPS (1µg) or
169 saline. Three days after immunization, mice were sacrificed and spleen cells
170 suspensions were obtained to study the proliferation of CD4⁺ T cells by flow cytometry.

171 *Trypanosoma cruzi protection assay*

172 *T. cruzi* antigens present in a whole homogenate (WH) of parasites were prepared from
173 epimastigote forms as described [14]. BALB/c mice were immunized s.c with 50µg of
174 WH alone or plus U-Omp19 (100µg) or CFA (100µl, first dose and IFA the second and
175 third doses) at day 0, 7 and 14. Three weeks after last immunization mice were injected
176 i.d with 1x10³ bloodstream trypomastigotes of RA strain. Parasitemia was monitored by

177 daily counting of the number of trypomastigotes per ml of fresh blood, and mortality
178 was recorded.

179 *Statistical analysis*

180 Statistical analysis and plotting were performed using GraphPad Prism 5 software
181 (GraphPad, San Diego, CA). In experiments with more than 2 groups, data were
182 analyzed using one-way ANOVA with Bonferroni's post-test. If necessary, a
183 logarithmic transformation was applied to obtain data with a normal distribution. When
184 bars were plotted results were expressed as mean $\pm$ SEM for each group. In survival
185 experiment Kaplan–Meier curve was generated using GraphPad Prism 5 software and
186 analyzed with log-rank test.

187 **Results**

188 *Subcutaneous U-Omp19 co-administration with OVA induces OVA-specific Th1* 189 *cellular immune responses*

190 To study if U-Omp19 has adjuvant properties when co-delivered subcutaneously
191 (s.c) with an Ag, OVA was used as model Ag. Thus, mice were s.c. immunized with
192 saline, OVA, OVA+U-Omp19 or OVA+CFA and three weeks later OVA-specific
193 cellular immune responses were evaluated. U-Omp19 co-administration with OVA
194 induced higher levels of IFN- γ production upon OVA stimulation compared with the
195 immunization with OVA alone ($P<0.01$ vs OVA immunized mice, **Fig. 1A**).
196 Immunization with CFA also induced production of IFN- γ by splenocytes ($P<0.001$ vs
197 OVA). In contrast, there were no differences in the levels of IL-4 or IL-10 produced by
198 spleen cells from the different groups (**Fig. 1B and C**). Furthermore, there was a slight
199 increment in IL-17 production by OVA-stimulated cells from U-Omp19+OVA

200 immunized mice but this increment was not statistically significant compared with the
201 production by cells from OVA-immunized mice (**Fig. 1D**). Immunization of mice with
202 U-Omp19 alone did not induce statistically significant production of cytokines after
203 OVA stimulation (data not shown). Con A induced the production of the corresponding
204 cytokines in all groups (data not shown). These results indicate that subcutaneous U-
205 Omp19 co-administration with OVA enhances OVA-specific Th1 cytokine responses.

206 Spleen cells from OVA+U-Omp19 or OVA+CFA immunized mice induced a
207 more potent T cell proliferation than the response elicited by splenocytes from OVA
208 administered mice ($P<0.05$, $P<0.001$ vs OVA, **Fig. 1E**). Moreover, U-Omp19+OVA
209 immunized mice showed an increment in the footpads thickness (DTH) compared with
210 mice immunized with OVA alone ($P<0.05$ vs OVA, **Fig. 1F**). Immunization with CFA
211 also induced a significant DTH response ($P<0.01$ vs OVA). Of note, U-Omp19 did not
212 induce any visible adverse effects on inoculated tissues as elicited by CFA inoculation
213 (**Fig. S1**). All together these results indicate that U-Omp19 s.c co-delivered with an Ag
214 induces Ag-specific Th 1 cellular immune responses *in vitro* and *in vivo*.

215 *U-Omp19 co-administration promotes Ag-experienced effector CD4⁺ T cells*

216 To determine whether T cells from immunized mice develop into memory T
217 cells or maintain an activated phenotype, the Ag-experienced T cells were identified by
218 the expression of CD44 [15, 16] in splenocytes from immunized mice after *in vitro* re-
219 stimulation. Memory T cells can be further divided based on CD62L (L-selectin)
220 expression into lymph node-homing T central memory cells (TCM, CD62L^{high}) and
221 tissue homing T effector memory cells (TEM, CD62L^{low}) [17]. Then, the percentage of
222 cells expressing CD44 and the activation marker CD62L was analyzed in CD4⁺ T cell
223 subset.

224 Phenotypic analysis of memory CD4⁺ T cells [15-17] showed that T cells from
225 mice s.c. administered with OVA+U-Omp19 exhibited a high proportion of the
226 CD44^{high} and CD62^{low} phenotype in CD4⁺ T cells (33.29%) indicating that a large
227 population of these cells are Ag-experienced cells compared with cells from mice
228 immunized with OVA alone (20.83%) and further indicates that Ag-experienced CD4⁺
229 T cells are maintained as effector, rather than central, memory cells in OVA plus U-
230 Omp19 immunized mice (**Fig. 2**). OVA+CFA immunization induced a similar profile of
231 effectors T cells to OVA+U-Omp19 immunized mice (31.43% CD44^{high} CD62^{low} CD4⁺
232 T cells, **Fig. 2**).

233 These data suggest that the vast majority of CD4⁺ T cells induced after Ag plus
234 U-Omp19 s.c. co-administration have a phenotype of activated, not resting, memory
235 cells.

236 *U-Omp19 subcutaneously co-administered with Ag alters Ag-specific IgG isotype profile*
237 *but does not enhance the magnitude of Ag- specific IgG humoral immune response*

238 No differences in the serum titer of OVA-specific IgG antibodies were found
239 after immunization with OVA alone or in combination with U-Omp19 (**Fig 3A**). As
240 expected, OVA+CFA vaccination increased significantly OVA-specific IgG titer in sera
241 ($P<0.001$ vs OVA, **Fig. 3A**). Of note, OVA+U-Omp19 modified the ratio between anti-
242 OVA IgG1 and IgG2a anti-OVA titers ($P<0.05$ vs OVA, **Fig. 3B**). While IgG1 titres
243 predominated over IgG2a in sera from mice injected with OVA or OVA+CFA, the
244 opposite was found in mice injected with OVA+U-Omp19. These results indicate that
245 U-Omp19 as adjuvant, delivered by s.c route, is not able to increase Ag-specific IgG
246 responses while it can change the elicited antibody subtype profile.

247 *U-Omp19 treated DCs promote IFN- γ production by specific CD4⁺ T cells and*
248 *increases T cell proliferation*

249 To study if U-Omp19 has the capacity to directly activate DCs and promote
250 effector specific CD4⁺ T cells responses, D011.10 mice were used as a source of OVA-
251 specific CD4⁺ T cells against OVA. Bone marrow derived dendritic cells (BMDCs)
252 from BALB/c mice were treated with OVA alone, OVA plus U-Omp19 or OVA plus
253 LPS for 24h, washed and then co-cultivated with CD4⁺ T cells from spleen DO11.10
254 mice for 3 days. DCs incubated with OVA+U-Omp19 were able to induce a higher level
255 of IFN- γ production in DO11.10 CD4⁺ T cells (11.1%) compared to T cells co-
256 cultivated with DCs pulsed with OVA alone (6.35%, **Fig. 4A**). LPS treated BMDCs
257 also induced a strong IFN- γ production by D011.10 cells (48.6%).

258 The priming capacity of U-Omp19 pulsed DCs on CD4⁺ T cells was also
259 assayed using a different Ag (chimeric GFP). Soluble chimeric GFP contains the MHC-
260 II restricted OVA peptide epitope (323-339, ISQAVHAAHAEINEAGR) recognized by
261 CD4⁺ OT-II cells (GFP-OTII). Of note, this chimeric protein is not internalized by
262 lectin-like receptors as OVA [13]. After co-incubation with GFP-OTII plus U-Omp19,
263 DCs were able to increase their Ag presentation to CD4⁺ T cells as revealed by a greater
264 percentage of IFN- γ producing OTII cells (1.96%) compared with DCs incubated with
265 GFP-OTII alone (0.49%, **Fig 4B**). In conclusion, U-Omp19 s.c. co-delivered with an Ag
266 promotes Ag presentation by DCs and the induction of IFN- γ producing CD4⁺ T cells *in*
267 *vitro*.

268 To determine *in vivo* the primary Ag-specific clonal expansion of T cells
269 following U-Omp19 s.c. administration, adoptive transfer assays using TCR transgenic
270 mice OTII were performed. Thus, C57BL/6 mice were adoptively transferred with

271 CFSE- labeled OT-II T cells. One day later, mice were s.c. immunized with OVA co-
272 administered with: U-Omp19, U-Omp19PK (protein digested with Proteinase K used as
273 control, **Figure 4D**), LPS or saline. Immunization with OVA plus U-Omp19 induced
274 greater OT-II T cell proliferation (61.7%) than immunization with OVA alone (31.7%,
275 **Fig 4C**). When U-Omp19 digested with proteinase K was co-delivered with OVA its
276 activity was lost since it induced a degree of proliferation (38.7%) similar to OVA
277 delivered alone. These results indicate that the measured activity resides in the protein
278 rather than to another non protein contaminant. As expected, a large percentage of
279 proliferating cells was obtained when LPS was co-administered with OVA (86.5%, **Fig**
280 **4C**).

281 *U-Omp19 subcutaneously co-administered with parasite derived antigens extends the*
282 *survival of mice after virulent parasite challenge*

283 Finally U-Omp19's adjuvant capacity using microbial derived Ags was
284 evaluated. A whole homogenate (WH) from *T. cruzi* parasites was used as Ag. Mice
285 were s.c inoculated with WH alone, WH plus U-Omp19 or WH emulsified in
286 Incomplete Freund Adjuvant (IFA) and 3 weeks later challenged with a lethal dose of
287 bloodstream trypomastigotes of the virulent RA strain. Mice s.c immunized with WH
288 plus U-Omp19 presented a significant reduction in weight loss and peak of parasitemia
289 when compared with animals that received the antigen alone after challenge (**Fig 5A**
290 **and B**, $P<0.05$). Most importantly, WH plus U-Omp19 immunization significantly
291 reduced mouse mortality when compared with immunization with WH alone (**Fig 5C**,
292 $P<0.05$). Immunization with WH plus IFA did not elicit a significant protective immune
293 response against parasite challenge (**Fig. 5A-C**).

294 Taken together these results indicate that U-Omp19 could be a suitable adjuvant
295 in vaccine formulations against pathogens in which protection is reached by promoting
296 Th1 immune responses.

297 **Discussion**

298 Adjuvants should be selected on the basis of the type of immune response that is
299 desired for a particular vaccine. In this work we demonstrated that U-Omp19 from
300 *Brucella* increases Th1 immune responses when is co-delivered s.c. with an Ag since it
301 increases *in vitro* Ag-specific T cell responses with IFN- γ production. Moreover, it
302 induces *in vivo* proliferation of CD4⁺ T cells and DTH responses after i.d challenge with
303 the Ag. Of note and in contrast to other known Th1 adjuvants -like CFA or CpG- U-
304 Omp19 co-administered s.c. does not increase antibody responses while is able to
305 change the isotype profile further indicating its immunomodulatory properties.

306 Induction of memory T cells is a key event in the generation of protective
307 immunity. *In vitro* stimulation of CD4⁺ T cells from mice s.c immunized with U-
308 Omp19 exhibits a larger percentage of effector memory T cells (CD4⁺ CD44^{high}
309 CD62L^{low} T cells). Central memory T cells expressing CD62L^{high} reside in the T-cell
310 zone of lymphoid organs and respond quickly to Ag re-stimulation proliferating and
311 differentiating into effector cells. Moreover, in this memory subpopulation the Ag
312 experienced T cells expressing the CD44 marker were increased when U-Omp19 is co-
313 delivered.

314 In previous reports we have shown that U-Omp19 can activate DCs *in vivo* by
315 up-regulating CD40, CD80 and CD86 [10]. In this work, *in vitro* assays indicate that U-
316 Omp19 treated DCs promote IFN- γ production by specific CD4⁺ T cells and increased

317 T cell proliferation indicating a direct effect on DCs Ag presentation. Immunization
318 with OVA plus U-Omp19 induced greater OVA-specific CD4⁺ T cell proliferation than
319 immunization with OVA alone. This activity is completely dependent on the presence
320 of the intact protein, since it was lost when the digested protein U-Omp19 (proteinase K
321 treated) was used as stimulant.

322 A common adverse effect using adjuvant compounds is their local reactogenicity
323 at the site of injection. Macroscopic analysis of the site of injection after s.c.
324 immunization with OVA plus U-Omp19 or plus CFA shows that CFA promotes the
325 formation of a pronounced and visible granuloma and inflammation signs while there
326 was not any visible local reaction after U-Omp19 administration at the injection site
327 **Fig. 6**). Besides, U-Omp19 s.c. co-administration does not have an effect in the
328 magnitude of the specific humoral response elicited but it has an effect on the isotypes
329 profile of this response. The generation of a humoral response balanced to the
330 generation of specific-IgG1 antibodies could be interesting to polarize immune
331 responses to a desired profile. For example, in many allergic processes turning the
332 allergen specific Th2 response to a modulatory Th1 response could be beneficial to
333 resolve or prevent allergic responses [18]. Indeed, one of the most accepted mechanisms
334 to explain the occurrence of autoimmunity following infection/vaccination is molecular
335 mimicry [19]. Thus, if using U-Omp19 the mentioned problem would not be expected.

336 The development of effective vaccine formulations against life-threatening
337 diseases especially those associated with poverty as well as neglected infectious
338 diseases is an actual scientific priority. In this work we have shown that U-Omp19 is a
339 suitable adjuvant to be included in future vaccine formulations against *T. cruzi* infection
340 since U-Omp19 s.c. co-delivered with whole homogenate (WH) from *T. cruzi* parasites

341 induced protection and significantly reduced mouse mortality. Of note, immunization
342 with WH plus IFA did not elicit a significant protective immune response against
343 parasite challenge. Further indicating the importance of evaluating different
344 formulations/adjuvants to the development of vaccines against infectious diseases.

345 Altogether the results presented in this work indicate that U-Omp19 delivered
346 subcutaneous could be a suitable adjuvant in vaccine formulations against pathogens in
347 which protection is mediated by Th1 cell immune responses.

348 **Acknowledgments**

349 The authors would like to thank Dr. Sergio Trombetta from New York University who
350 provided the GFP-OTII chimera and for his scientific advices and help.

351 This work was supported by grants from the Bill and Melinda Gates Foundation through
352 the Grand Challenges Explorations Initiative (OPP1017298 and OPP1060394); from the
353 Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT-Argentina): PICT
354 2010 N° 1163, PICT 2006 N° 1670, ANPCyT/CNPq PICT 2008 N° 18 (to Juliana
355 Cassataro).

356 **References**

- 357 [1] Reed SG, Orr MT, Fox CB. Key roles of adjuvants in modern vaccines. *Nat Med.*
358 2013;19:1597-608.
- 359 [2] Brito LA, Malyala P, O'Hagan DT. Vaccine adjuvant formulations: a pharmaceutical
360 perspective. *Seminars in immunology.* 2013;25:130-45.
- 361 [3] Alving CR, Peachman KK, Rao M, Reed SG. Adjuvants for human vaccines.
362 *Current opinion in immunology.* 2012;24:310-5.
- 363 [4] Harandi AM, Medaglini D, Shattock RJ, Working Group convened by E. Vaccine
364 adjuvants: a priority for vaccine research. *Vaccine.* 2010;28:2363-6.
- 365 [5] Moingeon P, Haensler J, Lindberg A. Towards the rational design of Th1 adjuvants.
366 *Vaccine.* 2001;19:4363-72.
- 367 [6] Seder RA, Hill AV. Vaccines against intracellular infections requiring cellular
368 immunity. *Nature.* 2000;406:793-8.

369 [7] Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends.
370 Immunol Cell Biol. 2004;82:488-96.

371 [8] Dougan G, Hormaeche C. How bacteria and their products provide clues to vaccine
372 and adjuvant development. Vaccine. 2006;24 Suppl 2:S2-13-9.

373 [9] Pasquevich KA, Estein SM, Garcia Samartino C, Zwerdling A, Coria LM,
374 Barrionuevo P, et al. Immunization with recombinant Brucella species outer membrane
375 protein Omp16 or Omp19 in adjuvant induces specific CD4+ and CD8+ T cells as well
376 as systemic and oral protection against Brucella abortus infection. Infect Immun.
377 2009;77:436-45.

378 [10] Pasquevich KA, Ibanez AE, Coria LM, Garcia Samartino C, Estein SM, Zwerdling
379 A, et al. An Oral Vaccine Based on U-Omp19 Induces Protection against B. abortus
380 Mucosal Challenge by Inducing an Adaptive IL-17 Immune Response in Mice. PLoS
381 One. 2011;6:e16203.

382 [11] Ibanez AE, Coria LM, Carabajal MV, Delpino MV, Risso GS, Cobiello PG, et al.
383 A bacterial protease inhibitor protects antigens delivered in oral vaccines from digestion
384 while triggering specific mucosal immune responses. Journal of controlled release :
385 official journal of the Controlled Release Society. 2015;220:18-28.

386 [12] Ibanez AE, Smaldini P, Coria LM, Delpino MV, Pacifico LG, Oliveira SC, et al.
387 Unlipidated outer membrane protein Omp16 (U-Omp16) from Brucella spp. as nasal
388 adjuvant induces a Th1 immune response and modulates the Th2 allergic response to
389 cow's milk proteins. PloS one. 2013;8:e69438.

390 [13] Drutman SB, Trombetta ES. Dendritic cells continue to capture and present
391 antigens after maturation in vivo. Journal of immunology. 2010;185:2140-6.

392 [14] Segura EL, Vazquez C, Bronzina A, Campos JM, Cerisola JA, Cappa SM.
393 Antigens of the subcellular fractions of Trypanosoma cruzi. II. Flagellar and membrane
394 fraction. J Protozool. 1977;24:540-3.

395 [15] Dutton RW, Bradley LM, Swain SL. T cell memory. Annual review of
396 immunology. 1998;16:201-23.

397 [16] Pure E, Cuff CA. A crucial role for CD44 in inflammation. Trends in molecular
398 medicine. 2001;7:213-21.

399 [17] Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory
400 T lymphocytes with distinct homing potentials and effector functions. Nature.
401 1999;401:708-12.

402 [18] Cramer R, Rhyner C. Novel vaccines and adjuvants for allergen-specific
403 immunotherapy. Curr Opin Immunol. 2006;18:761-8.

404 [19] Cusick MF, Libbey JE, Fujinami RS. Molecular mimicry as a mechanism of
405 autoimmune disease. Clinical reviews in allergy & immunology. 2012;42:102-11.

406

407

408

409

410

411

412 **Figure legends**

413

414 **Figure 1. U-Omp19 s.c. co-administration with an Ag increases Ag-specific Th1**
415 **immune responses in BALB/c mice.** Mice ($n=5/\text{group}$) were subcutaneously
416 administered with OVA, OVA+U-Omp19, OVA+CFA or saline. Spleens from mice
417 were obtained 3 weeks after the last immunization. Cytokine production was determined
418 by ELISA on supernatants harvested 5 days after stimulation of spleen cells with OVA
419 or complete medium. Production of IFN- γ (A), IL-4 (B), IL-17 (C) and IL-10 (D) is
420 shown. Results are presented as mean $\pm$ SEM for each group and are representative of 3
421 independent experiments. $*P<0.05$, $**P<0.01$, $***P<0.001$ vs OVA group. Delayed-
422 type hypersensitivity (DTH) response to OVA (E) was assayed 3 weeks after the last
423 boost to evaluate the cellular immune response *in vivo*. Twenty μg of OVA were
424 injected into one footpad, and saline was injected into the contra lateral footpad, as a
425 negative control. The thickness of both footpads was measured 48 h later and the
426 increment between OVA and saline injected was plotted. ($*P<0.05$, $**P<0.01$ vs OVA
427 group). T-cell proliferation from immunized mice (F). Splenocytes were stimulated *in*
428 *vitro* with OVA (100 or 1000 $\mu\text{g}/\text{ml}$) for 5 days and proliferation was assessed by
429 thymidine labeling. The number of counts per minute (cpm) was determined. Data are
430 presented as mean $\pm$ SD. ($*P<0.05$, $***P<0.001$ vs OVA group).

431

432 **Figure 2. U-Omp19 as adjuvant by s.c route promotes memory Th1 cellular**
433 **immune responses.** Phenotypic analysis of memory T cells by flow cytometry.
434 Animals were immunized as described in figure 1. Splenocytes from immunized mice
435 were stimulated with the Ag *in vitro* for 5 days. Percentage of CD62L and CD44

436 markers expression in CD4 T cells was determined by flow cytometry. Subpopulations
437 are shown in dot plots and proportions are shown in pie chart below.

438

439 **Figure 3. U-Omp19 delivered s.c. does not increase IgG humoral response against**
440 **co-administered Ag.** Animals were immunized as described in figure 1. Specific-OVA
441 IgG, IgG1 and IgG2a titers were determined by ELISA in serum from immunized mice.
442 * $P < 0.05$, *** $P < 0.001$ vs OVA group.

443

444 **Figure 4. U-Omp19 promotes Ag presentation by DCs *in vitro*.** (A) Production of
445 IFN- γ by D011.10 CD4⁺ T cells. BMDCs were incubated with OVA alone, plus U-
446 Omp19 or LPS for 20hs and then washed and co-incubated with DO11.10 T cells for 3
447 days. Determination of intracellular IFN- γ in CD4⁺ T cells was evaluated by flow
448 cytometry. IFN- γ producing CD4⁺ T cells frequencies is shown. (B) Production of IFN-
449 γ by OTII CD4⁺ T cells. BMDCs were incubated with GFP-OTII chimera protein alone,
450 plus U-Omp19 or LPS for 20hs and then washed and co-incubated with OTII T cells.
451 Intracellular IFN- γ staining was performed and the percentage of IFN- γ producing OTII
452 T cells was determined by flow cytometry. (C) Proliferation *in vivo* of OTII CFSE
453 labeled T cells after s.c. administration of i) saline, ii) OVA, iii) OVA+U-Omp19, iv)
454 OVA+ U-Omp19PK or v) OVA+LPS. Three days later spleen cells were harvested and
455 cell suspensions were analyzed by flow cytometry. Results are shown as representative
456 histograms and data represented as percentage of OTII CD4⁺ CFSE⁺ proliferative T
457 cells ($n=3$ /group). Representative of three different experiments. (D) Degradation of U-
458 Omp19 with Proteinase-K. U-Omp19 was treated with proteinase K-agarose from
459 *Tricirachium album* for 1h at 37°C. The enzyme immobilized in agarose was then
460 centrifuged out and the supernatants were incubated for 2h at 60°C to inactivate any

461 fraction of soluble enzyme. The complete digestion of U-Omp19 was checked by SDS-
462 PAGE followed by Western Blot analysis. Line 1: Molecular weight marker, line 2: U-
463 Omp19, line 3: U-Omp19 PK.

464

465 **Figure 5. U-Omp19 s.c. co-administration induces protection against *T. cruzi***
466 **challenge.** BALB/c mice ($n/\text{group}=7$) were s.c. immunized at days 0, 7 and 14 with i)
467 whole homogenate (WH) from *T. cruzi* parasites, ii) WH+U-Omp19 or iii) WH+IFA.
468 Three weeks after last boost mice were i.p. challenged with bloodstream
469 trypomastigotes (RA strain). Weight loss, blood parasite levels and survival were
470 monitored until day 30 after challenged. Data are presented as mean of grams of weight
471 loss per group (**A**), parasites/ml per mouse at the peak of parasitemia (**B**) and percent of
472 survival of mice (**C**). $\ast P<0.05$ vs WH group.

473

474 **Figure 6. U-Omp19 does not induce any visible adverse effect at the site of**
475 **injection.** BALB/c mice were s.c. immunized at the right flank with OVA+U-Omp19 or
476 OVA+CFA at day 0, 7 and 14. Three weeks after last immunization pictures were
477 captured to determine any visible inflammation signs in the tissues at the site of
478 infection. Representative pictures are shown.

479

figure 1

figure 2

figure 3

figure 4

figure 5

figure 6