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Mucosal and systemic immune responses induced by a Single Time Vaccination Strategy in mice

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Manuscripts

1 **Mucosal and systemic immune responses induced by a Single Time Vaccination Strategy in**
2 **mice**

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23 ***Abstract***

24 Vaccination is considered by World Health Organization as the most cost-effective strategy for
25 controlling infectious diseases. In spite of great successes with vaccines, many infections
26 diseases are still leading killers, because of their inadequate coverage. Several factors have been
27 responsible: number of doses, high vaccine reactogenicity, vaccine costs, vaccination policy,
28 among others. Contradictorily, few vaccines are of single dose and even less of mucosal
29 administration. However, more common infections occur via mucosa, where secretory
30 immunoglobulin A plays an essential role. As an alternative, we proposed a novel protocol of
31 vaccination called Single Time Vaccination Strategy (SinTimVaS) by immunizing two priming
32 doses at same time, one by mucosal and one by parenteral routes. Here, the mucosal and
33 systemic responses induced by Finlay Adjuvants (AF Proteoliposome (PL) 1 and AF Cochleate
34 (Co) 1) implementing SinTimVaS in Balb/c mice were evaluated. One intranasal dose of AFCo1
35 and intramuscular dose of AFPL1 adsorbed onto Aluminum hydroxide, with BSA or TT as
36 model antigens, administrated at the same time, induced potent specific mucosal and systemic
37 immune responses. Also, we demonstrated that SinTimVaS using other mucosal routes like oral
38 and sublingual, in combination with subcutaneous route elicits immune responses. SinTimVaS,
39 as a new immunization strategy, could increase vaccination coverage and reduce time-cost
40 vaccines campaigns, adding the benefits of immune response in mucosa.

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42 Keywords: adjuvants, mucosal immunization, vaccination strategy, cochleates, outer membrane
43 vesicles

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47 **Introduction**

48 Vaccination is considered by the World Health Organization (WHO) as the most cost-effective
49 strategy for controlling infectious diseases that prevent 2.5 million of deaths every year (WHO,
50 2005). Despite of the great success of vaccination, many infectious diseases are still leading
51 killers, mainly due to their insufficient vaccination coverage (WHO, 2009; Drain et al. 2003). A
52 number of factors have been responsible of the logistical challenges to attain high immunization
53 coverage: required doses on immunization schedules, parenteral immunization, high vaccine
54 reactogenicity, few studies targeting antigens-combinations on vaccine formulations, vaccine
55 cold chain, vaccination policy, countries weak immunization programmes, high cost and not
56 affordability for poor countries, among others (Jiang et al. 2005). Only live-attenuated vaccines
57 like BCG and Polio induce good protection with a single dose. In contrast, to obtain good
58 immune response with a single dose by non-living antigen vaccines multiple doses are generally
59 needed, in order to provide sufficient stimulation of the immune system and to achieve long-
60 lasting immune responses. The number of doses is especially relevant in developing countries,
61 where the population has not easy access to medical services and vaccination campaigns are
62 very difficult, much more when several doses are required, which cause loses the entire
63 immunization schedule (Aguado and Lambert 1992). So, the development of new immunization
64 strategies that allow complete immunization scheme with a single visit without further contact
65 could be as important as to develop single-dose vaccines.

66 Traditionally, adjuvants have been used to enhance the magnitude of the adaptive response to a
67 vaccine. However, a second role has become increasingly important: guiding the type of adaptive
68 response to produce the most effective forms of immunity for each specific pathogen (Th1, Th2,
69 Th17, Th9) (Coffman et al. 2010). Adjuvants have been classified as: immunopotentiators,
70 delivery systems (O'Hagan and Singh 2003), and immunopolarizators(Perez et al. 2013) and
71 neither of these activities are mutually excluding, even, some adjuvants exhibit at least two of

72 them. The use of potent adjuvants that exhibit several mechanisms of action and enabling
73 comparable immune responses using substantially lower antigen amount and doses could be very
74 important to develop novel vaccination strategies. AFPL1 (Adjuvant Finlay Proteoliposome 1), a
75 nanoparticle derived from the outer membrane of *Neisseria meningitidis* serogroup B, has been
76 widely used as the main antigen of the Cuban meningococcal vaccine administered in more than
77 60 million doses in humans (Campa et al. 1997 and Perez et al. 2001). AFCo1 (Adjuvant Finlay
78 Cochleate 1), is a microparticle with cochlear structure obtained from the interaction of anionic
79 lipid from AFPL1 with divalent cations, like Ca^{2+} (Perez et al. 2004)). Both structures combine
80 in the same structure: 1) immunopotentiator activity, containing major outer membrane proteins
81 including Por B, lipopolysaccharide (LPS), phospholipids and others synergistic microorganism-
82 associated molecular patterns (MAMPs) like traces of DNA; 2) immunopolarizer actions,
83 inducing a preferential Th1 pattern; and 3) delivery system capacity, based on lipids presence
84 (Del Campo et al. 2009). Their potential as adjuvants has been tested with a variety of antigens:
85 *Plasmodium falciparum* merozoite surface protein 4 and 5 (Bracho et al. 2009), Herpes Simplex
86 Virus type 2 protein (Del Campo et al. 2010), *Streptococcus pyogenes* proteins (Guillherme et al.
87 2009) and purified *Dermatophagoides siboney* allergens (Lastre et al. 2006). Both structures also
88 induce cross-presentation, and cytotoxic T-lymphocyte activity (CTL) (Rodriguez et al. 2005 and
89 Rodriguez et al. 2006). AFCo1 can be used mainly as mucosal adjuvant because it is a stable
90 particle with higher mucosal absorption (Del Campo et al. 2009) and acid resistant properties.

91 Mucosal surface is exposed to a diverse and very large number of microorganisms and also is the
92 site of infection of diverse pathogens (e.g., influenza virus, *Vibrio cholerae*, type I reovirus, and
93 rhinovirus) (Plotkin, 2005). The initial mucosal infection can only be prevented by mucosal
94 immune responses (pathogen-specific secretory IgA (SIgA) or local cytotoxic T lymphocytes
95 (CTL)) (Brandtzaeg, 2009). Mucosal immunization elicits systemic specific immune response
96 and stimulates the specific mucosal immune response, leading by the production of SIgA

antibody. Also, it is less invasive route; does not require trained personnel; and significantly decreases the risk of parenteral transmitted diseases (Mann et al. 2009; Neutra and Kozlowski, 2006). In our experience mucosal route requires larger antigen amount and number of doses than parenteral route to obtain similar systemic responses (Del Campo et al. 2009). In addition, parenteral immunization has been shown induction of memory IgA in different vaccines, such as influenza, *Pseudomonas aeruginosa*, and inactivated polio vaccines (attributed to previous natural mucosal infection by these organisms) (Kaul and Ogra, 1998). Indeed, a combined mucosal and parenteral poliovirus immunization strategy has been shown to effectively induce high neutralizing antibodies in serum as well as mucosal immune responses in humans. Strategies which combine together parenteral and mucosal immunizations (Treanor et al. 1992; Morrison et al. 1998), such as mucosal prime-parenteral booster (Lee et al. 1999; Faden et al. 1990) or parenteral prime-mucosal booster (Keren et al. 1988) have generated stronger immune responses than either of these routes by themselves.

In the light of the ambitious goal of vaccines providing maximum protection at both parenteral and mucosal levels with minimum number of doses; we set out to develop a novel vaccination strategy, called Single Time Vaccination Strategy (SinTimVaS) (Perez et al. 2008; Gonzalez et al. 2009). SinTimVaS combines two priming (one mucosal and one parenteral) at the same time without subsequent boost; being the presence of potent adjuvants one of the primary goals. In this work, we assessed the ability of SinTimVaS to induce an efficient systemic and mucosal immune response in mice against bovine serum albumin (BSA) protein and Tetanus Toxoid (TT) by different mucosal and parenteral immunizations routes. We showed that mice immunized with one AFCo1 intranasal (i.n) dose and one AFPL1 intramuscular (i.m) dose at the same time combined with BSA or TT antigens triggered specific serum IgG and mucosal IgA immune response against these specific antigens. Furthermore, we demonstrated that the new strategy also works for oral (o.r), sublingual (s.l), and subcutaneous (s.c) routes combinations.

122

123 **Materials and methods**124 **Animals**

125 The experiments were assessed using female Balb/c mice from National Center for the
126 Production of Laboratory Animals (CENPALAB), Havana, Cuba of 8-10 weeks of age and 16-
127 18 g of weight. Mice were randomly distributed into five mice *per* experimental groups or
128 control groups. All mice were kept in ventilated cages, water ad libitum under specific conditions
129 at the Finlay Institute Animal Research Facilities. All animal studies were carried out following
130 the guidelines for the use of Laboratory Animals (EEC Council Directive, 1987) and were
131 approved by the Ethics Committee for Laboratory Animals in Havana, Cuba.

132 **Adjuvants and antigens**

133 TT and AFPL1, of the epidemic meningococcal strain Cu385 (B:4:P1.19,15), were produced at
134 industrial scale under Good Manufacturing Practices (GMP) conditions by manufacturing plants
135 at Finlay Institute, Havana, Cuba (9). AFPL1 was absorbed onto aluminium hydroxide (Alum).
136 AFCo1 was obtained in our laboratory starting from PL, through a process of exchange of
137 sodium deoxycholate (DOC) and the divalent cation calcium as described by Pérez *et al* (8). The
138 formation of cochleate structure was visualized by the appearance of a white suspension in the
139 preparation and by light microscopy analysis. BSA protein was supplied from Sigma, Aldrich.

140 **Immunization schedules**

141 Groups of 5 female mice were immunized simultaneously by i.m injection of AFPL1 (12.5 µg)
142 or by mucosal (i.n, s.l, or o.r) administration of AFCo1 (50 µg). Also, groups of mice were
143 immunized 2 times (days 0 and 14) i.m with AFPL1 (12.5 µg), and 3 times (days 0, 7 and 14)
144 i.n (12.5 µL/nostril) with AFCo1 (50 µg). With heterologous antigens, mice were immunized
145 using the same scheme described above. Each dose contained BSA (10 µg per mouse per dose)
146 plus AFPL1 (12.5 µg) or TT (2Lf (limit to flocculation) per dose per mouse) plus AFPL1 (12.5

147 μg) on i.m or s.c immunizations. In i.n, s.l and o.r immunizations, each dose contained BSA (20
148 μg per dose per mouse) or TT (5 Lf per dose per mouse) plus ACo1 (50 μg). The volume
149 delivered in each dose was: 12.5 μL *per* nostril for i.n, 10 μL under the tongue for s.l, and 100
150 μL for o.r routes. Groups of animals receiving saline were used as experimental control for
151 immunization. Table 1 shows the different SinTimVaS combinations used.

152

153 **Samples collection**

154 Samples were collected as follows. saliva and faeces were collected on the 7 day after the last
155 dose, serum and vaginal washes on the 21st days after the last dose (12-30). Saliva samples were
156 taken following salivation stimulation with 50 μL intraperitoneal injection of pilocarpine (0.5%
157 (v/v) Quimefa, Cuba). Saliva was inactivated for 15 min at 56°C, centrifuged at 10000 g for 10
158 min and stored until it was used. Three to 6 pieces freshly voided faeces were collected into 1.5
159 mL pre-weighed micro-centrifuge tubes and phosphate buffered saline (PBS, pH 7.2) was added
160 with protease inhibitors (1 mM phenylmethyl sulfonylfluoride in ethanol, 5 $\mu\text{g}/\text{mL}$ of aprotinin
161 and 1 $\mu\text{L}/\text{mL}$ of leupeptin, antipain and pepstatin (all from Sigma) in a ratio of 20 μL per mg of
162 faeces. Solid matter was resuspended by extensive vortex and centrifuged at 10000 g for 10 min
163 and the supernatants were stored. Vaginal cavities were rinsed 3 times with 50 μL of PBS,
164 centrifuged and immediately stored. Blood was collected by puncturing the retro-orbital plexus
165 using microhematocrit capillary tubes. The serum was removed by centrifugation at 5000g for 10
166 min. All samples were stored at -20°C until ELISA's assays were conducted.

167

168 **Analysis of immune response by Enzyme-linked immunosorbent assay (ELISA)**

169 Anti PL, BSA, or TT-specific IgA, IgG, IgG1, and IgG2a antibodies were measured. Briefly, 96-
170 well ELISA plates (Nunc MaxiSorp F96; Roskilde, Denmark) were coated overnight at 4°C with
171 100 $\mu\text{L}/\text{well}$ with: PL (20 $\mu\text{g}/\text{mL}$), BSA (10 $\mu\text{g}/\text{mL}$) or TT (5 Lf/mL) in carbonate bicarbonate

172 buffer (pH 9.6). After blocking with PBS-1% Gelatin (Blocking Solution (BS)) and incubated at
173 room temperature for 1 hour, plates were washed with PBS containing 0.05% Tween-20. Then,
174 samples were diluted in BS as following: 1:2 for saliva and feces, 1:10 for vaginal washes and
175 1:100 for sera, adding 100 μ L/well and incubated 2h at 37°C. The plates were incubated at 37°C
176 for 2 h with the different conjugates: anti-mouse IgA biotinylated antibody (4 μ g/mL, Sigma,
177 USA), anti-mouse IgG peroxidase-conjugated antibodies (1:5000, Sigma, USA), and anti-mouse
178 IgG1 and IgG2a biotinylated antibody (1:2500, Sigma, USA) for IgG isotypes. Streptavidin
179 peroxidase-conjugate (1:2000 dilution, Sigma, USA) was added and incubated for 2 h at 37°C.
180 Each plate was developed with O-phenylene diamine (OPD) (Sigma, USA) in citrate-phosphate
181 buffer pH 5.0 and the reaction was stopped with H₂SO₄ 2 M. Optical densities (OD) were read
182 at 492 nm and the results were expressed as OD. Anti PL IgG and IgA titers were expressed as
183 arbitrary units per mL (AU/ mL) referred to an in-house standard. The cut-off value was
184 established as the mean OD 492 plus two times the standard deviation (SD) of control group. In
185 all plates a positive and negative sera were included to validate the assay.

186

187 **Statistical analysis**

188 Data were analyzed using the GraphPAD prism software (San Diego, CA, USA). The statistical
189 significance of the difference between group means was analyses by Student's two-tailed *t*-test
190 for two groups, or by one-factor analysis of variance (ANOVA) followed by Tukey's test for
191 three or more groups. Differences were considered significant with $P < 0.05$.

192

193 **Results**

194 **SinTimVaS applied by intranasal / intramuscular routes induces anti PL mucosal and**
195 **systemic immune responses**

196 To determinate if SinTimVaS (i.n/i.m) might induce systemic and mucosal immune response,
197 this novel strategy with the traditional immunization schedule of 3 i.n and 2 i.m doses were
198 compared. Groups of Balb/c mice were primed mucosally (i.n) and systemically (i.m) at the
199 same time without subsequent booster. As AFCo1 and AFPL1 have conserved in their structure
200 several protective outer membrane proteins, we can determinate immune responses against their
201 self. Twenty one days after the last dose, the levels of systemic and mucosal immunity were
202 evaluated by determining serum anti PL IgG and IgA responses. The serum anti PL IgG induced
203 by SinTimVaS were similar to those induced by 3 i.n doses of AFCo1 or 2 i.m doses of AFPL1
204 (Fig. 1A), maintaining the same subclasses behaviour (Fig. 1B).. Thus, the observed levels of
205 IgG2a in mice immunized by different SinTimVaS combinations suggest a Th1 polarization
206 (Fig. 2B) as is the case of the immune response of mice immunized with 3 i.n or 2 i.m doses. In
207 saliva, anti-PL IgA was significant higher ($p<0.001$) than 2 i.n or 2 i.m doses of AFCo1 and
208 AFPL1, respectively. In addition, neither one i.n nor 2 i.m doses induce specific IgA (Fig. 1C).
209 Overall, SinTimVaS overcome the several required doses for mucosal immune response
210 induction when it is supplemented with a simultaneous parenteral dose.

211 **SinTimVaS works with other mucosal routes**

212 In order to evaluate if SinTimVaS can be extended to other mucosa routes, s.l/i.m and o.r/i.m
213 combination were administrated and compared to traditional immunization schedules.
214 SinTimVaS combination of s.l/i.m or o.r/i.m priming induce systemic immune response
215 significant higher ($p<0.01$) than 3 s.l and 3 o.r doses of AFCo1 (Fig. 2A). We also tested specific
216 IgG subclasses in the sera of immunized mice, and found that IgG1 and IgG2a were induced in
217 all SinTimVaS combinations (Fig. 2B). In addition, the presence of high levels of IgG2a in sera
218 constitutes direct evidence of Th1. The anti PL IgA response was detected in all mucosal
219 samples (saliva, feaces and vagina). Both SinTimVaS combinations (s.l/i.m and o.r/i.m) induce
220 similar mucosal specific IgA in all mucosal samples when we compared with 3 s.l and 3 o.r

221 doses of AFCo1. Nevertheless, anti-PL specific IgA response induced by i.n route is higher to
222 other mucosal routes (Fig. 2C). Lastly, mucosal specific IgA was induced not only at the regional
223 site of immunization but also at distal places like the vagina and intestine when i.n and s.l routes
224 were used in SinTimVaS combinations. These results confirmed that SinTimVaS works even
225 when other mucosal routes of immunization were used.

226 **Mucosal and systemic immune response induced by SinTimVaS with subcutaneous route**
227 **as parenteral priming.**

228 To determinate whether the s.c route as parenteral priming in SinTimVaS induces systemic and
229 mucosal immune responses, we compared different combinations of mucosal (i.n, s.l, o.r) routes
230 and s.c route with 3 mucosal doses of AFCo1 and 2 s.c doses of AFPL1(Fig. 3). At systemic or
231 mucosal levels not differences were observed when s.c priming was used. Also, all SinTimVaS
232 combinations were capable to induce high levels of IgG1 and IgG2a. These results demonstrated
233 that this novel strategy of immunization also works with s.c route as parenteral priming.

234 **Mucosal and systemic immune responses induced by SinTimVaS when AFPL1 and AFCo1**
235 **were used as adjuvants against BSA and TT.**

236 To address if Finlay Adjuvants (AFPL1, to parenteral routes and AFCo1 to mucosal routes) are
237 able to elicit immune response against two model antigens (BSA and TT) using the proposed
238 strategy, mucosal and systemic anti-BSA and anti-TT immune responses were examined..
239 Figure.4A and B show anti-BSA specific IgG in sera and IgA in saliva, respectively.
240 SinTimVaS i.n/i.m combination induced superior response anti-BSA IgG ($p<0.05$) than s.l/im or
241 o.r/i.m combinations (Fig. 4A). Also, the response at the systemic level in all SinTimVaS
242 combinations were significantly superior ($p<0.05$) to 3 doses of each mucosal route. Figure 4B
243 show that we detected a slight increase in anti-BSA IgA response in mice immunized with
244 i.n/i.m and o.r/i.m combinations when compared with each only-mucosal scheme, respectively.
245 Figure 4C shows anti-TT systemic response induced by SinTimVaS combinations.. Furthermore,

the anti-TTspecific IgG in sera of o.r/i.m and s.l/i.m combinations was stimulated in comparison with antibodies levels induced by 3 oral or sublingual doses. Moreover, anti-TT IgG response induced by i.n/i.m combination was similar to those induced by 3i.n doses of AFCo1-TT or 2 i.m doses of AFPL1-TT. However, in saliva anti-TT response of SinTimVaS combinations was not superior to the response induced by 3 doses of AFCo1-TT by different routes (Fig. 4D), but it was significantly higher ($p<0.05$) than the IgA detected in mice immunized with 3 doses of TT alone (data not show). These results indicate that Finlay adjuvants in SinTimVaS are highly effective in eliciting the production of both mucosal and systemic immune responses against heterologous antigens.

Discussion

SinTimVaS constitutes a novel alternative to solve the problems of current catch-up vaccines campaigns, which are so difficult to implement in large countries with weakened health systems. Simultaneous immunization of one mucosal dose (i.n, s.l, o.r) and one parenteral dose (i.m, s.c) as the complete immunization scheme induces efficient systemic and mucosal responses, similar to those obtained by two only-parenteral or three only-mucosal immunizations, respectively. . The Global Immunization Vision and Strategy 2006-2015, elaborated by WHO Department of Immunization, proposed to carry out in a short-term period, the introduction of new vaccines, technologies and strategies, focusing on the need to increase vaccination coverage and reduce the morbidity and mortality from infectious diseases. More research on less expensive immunization strategies should be prioritized on the view to reducing cost-effectiveness of vaccines. Sustainable systems for children, adolescents, and adults vaccination must be developed (CDC, 2005). In this sense, the importance of SinTimVas lies on the possibility for providing better vaccination coverage: achieving that in a single visit, the full immunization schedule is covered and booster doses are not needed. Our vaccination regime induced high titers of IgG2a, even comparable to the levels obtained when mice were vaccinated with the 3 nasal doses of AFCo1

271 or 2 i.m doses of AFPL1. An induction of IgG2a antibodies generally indicates Th1 immunity,
272 whereas the induction of IgG1 antibodies is easier to induce and is not a conclusive indication of
273 a Th2 response.

274 Initial work of a consecutive immunization strategy, known as "prime-boost" were developed
275 with DNA vaccines; involving priming with DNA and boosting with vaccines encoding multiple
276 common HIV antigens. Prime-boost vaccination strategies amplify T-cell immunity and
277 ameliorate AIDS in macaques (Dale et al. 2006). In our study, the combination of only-one
278 parenteral dose (i.m or s.c) reinforced by a simultaneous subsequent mucosal dose, induce
279 efficient mucosal immune response. The levels of with secretory IgA were as high as the
280 sequential mucosal doses (one week apart from each other). Additionally, the use of other
281 mucosal routes (s.l or o.r) in the strategy elicited strong systemic and mucosal responses, with
282 SIgA detected in saliva, faeces or vaginal washes. Furthermore, it is known that AFPL1 can
283 activate the maturation of dendritic cells (DC) and macrophages (Rodriguez et al. 2005). Thus,
284 the maturation of DC might have contributed to the strong mucosal responses detected. Vajdy y
285 cols., 2003 found that oral or intranasal followed by i.m. immunizations induced significantly
286 higher serum titres against *H. pylori* antigens (NAP and CagA) compared to i.n. alone, oral alone
287 or i.m. alone (Vajdy et al. 2003). More importantly, they found that while both i.n. and i.m. recall
288 immunizations induced similar levels of serum antibody responses, mucosal IgA responses in
289 faeces were only achieved through i.n. recall immunization (Vajdy et al. 2003).

290 This novel vaccination approach also showed that mice immunized by subcutaneous route can
291 induce potent antibody responses at mucosal and systemic compartments. However, in the s.c
292 route the antigen is administrated to the adipose tissue while in the i.m route the antigen delivery
293 is directly to the muscle, which allows a sustained absorption of the antigen. Patrolling DCs are
294 also numerous in the well-vascularized muscles and they are fewer in adipose tissues (Balasse et
295 al. 2008), therefore it could be hypothesized that i.m injection in SinTimVaS may be considered

296 as a priming dose. . Moreover, both adjuvants have potent activity as immunopotentiators, given
297 by the MAMPs presented in their structures. The recognition of these MAMPs by TLRs, leads to
298 activation of a complex system of signal transduction, leading to the maturation and activation of
299 DC. Previous studies by our group have demonstrated the potent DC activation by the AFPL1,
300 evidenced by a high production of pro-inflammatory cytokines (IL-1 β , IL-6, TNF and IL-12)
301 (Perez et al. 2001) and by the high expression in DC of co-stimulatory molecules CD40, CD80
302 and CD86 (Rodriguez et al. 2005). Immunized MyD88 deficient mice with AFCo1 and
303 glycoprotein D of HSV-2 by intranasal route were unable to produce specific proliferative and
304 IFN- responses of splenic CD4⁺ cells. The presence of TLR adaptor molecule MyD88 has
305 demonstrated to be necessary to induce a potent IgG response and protect against challenge with
306 herpes simplex virus type 2 (HSV-2) (Del Campo et al. 2010). AFCo1 and AFPL1, contains
307 major outer membrane proteins of *N. meningitidis* B, including LPS and porins (PorA and PorB)
308 (Del Campo et al. 2009) that can activate TLR4 and TLR2, respectively. While TLR2 transduces
309 signal exclusively through the adaptor molecule MyD88, LPS exerts its immunopotentiating
310 effect through a MyD88-dependent as well as a MyD88 independent TIR-domain-containing
311 adapter-inducing interferon- (TRIF)-based pathways (Perez et al. 2012).

312 More importantly, we have shown that SinTimVas is capable of activating immune response
313 against heterologous antigens when Finlay Adjuvants are incorporated in the combination. The
314 SinTimVaS approach induced high specific anti-heterologous antibodies titers in sera and
315 mucosal compartments similar to two-parenteral weekly spaced doses or several weekly spaced
316 mucosal doses. It is well known that parenteral immunization usually elicits a peripheral immune
317 response, with protective IgM/IgG antibodies and peripheral cell-mediated immunity but fails to
318 stimulate mucosal lymphatic tissues to generate protective IgA antibodies (Lawson et al. 2011).
319 Meanwhile, mucosal immunization confers immunity at the directly vaccine-exposed mucosa
320 followed by the adjacent mucosal tissues. Also, mucosal tissues are interconnected *via*

321 circulating lymphocytes; immunization at one mucosal inductive site may induce an immune
322 response at other, even remote, mucosal surface (Puga et al. 2010). The mucosal efficacy of
323 AFCo1 appears to owing a directly engagement with B cells or other antigen presenting cells.
324 Direct TLR stimulation enhances T independent IgA class switching. As the antigens immunized
325 by parenteral routes reach the lymphatic pathway earlier than those who are immunized by
326 mucosal routes, we can hypothesize that in SinTimVaS, mucosal immunization can works as a
327 booster of the parenteral immunization.

328 Small-sized particles as vaccine delivery systems are efficient in permeating biological barriers
329 and affecting the kinetics of lymphoid drainage (Singh et al. 2007). In our strategy, a
330 combination of nanoparticle and microparticle was used simultaneously. AFPL1, a nanometric
331 particle of 70 nm was applied by parenteral immunization. Nanoparticles of less than 200 nm
332 reach the lymphoid organs directly through the lymph drainage within hours of injection,
333 whereas the transport of particles larger than 200–500 nm requires DCs, and it takes
334 approximately 24 hours for them to arrive in lymph nodes (Jennings et al. 2007; Bachmann and
335 Jennings, 2010). On the other hand, AFCo1, a microparticle with $\approx 11\mu\text{m}$ of length, was applied
336 by mucosal route. There are reports showing that particles in the size range of 2–8 μm were
337 capable to induce after a single-point i.m immunization high antibody titers, whereas particles of
338 50–150- μm of length generated weaker antibody titers (Bachmann and Jennings, 2010). Also,
339 data from other studies reported considerable antibody responses even from large microparticles
340 of 10–90- and 15–60- μm size ranges (41). Finally, results from our group have shown that
341 SinTimVaS using ovalbumin as model antigen is capable to induce systemic anti-Ova IgG
342 response and long-lasting memory response, when a booster of ovalbumin at day 125 was
343 administrated (Perez et al., 2013).

344 In summary, two priming applied at the same time by different immunization routes can induce
345 efficient mucosal and systemic responses. Undoubtedly, the strength of the new vaccination

346 strategy (SinTimVaS) is to improve vaccination coverage, reducing the logistics and the number
347 of follow-up and catch-up campaigns. SinTimVaS could solve non-continuity vaccination
348 problems, allowing a complete - vaccination scheme in one immunization service. Further
349 studies to explain the mechanisms involved in the activation of the immune response by this
350 strategy are needed. Greater understanding of SinTimVaS mechanism to increase and sustain
351 immunization levels is necessary in order to create long-lasting responses. Other issues to
352 consider are the application of SinTimVaS to other parenteral and mucosal adjuvants and to
353 evaluate long-lasting immune responses against other antigens.

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360

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Figure 1. PL-specific antibody responses induced by SinTimVaS. Groups of female BALB/c mice (n = 5) were SinTimVaS (i.n/i.m) immunized with AFPL1 (i.m route), AFCo1 (i.n route). Control groups included were IN, IM immunized with one, two or three doses with AFPL1 or AFCo1. The PL-specific IgA in their saliva, IgG levels and IgG subtypes in sera were determined using a PL-specific ELISA. (A) Shows the anti-PL IgG levels in sera (B) Shows anti-PL IgG subclasses in sera (C) Shows the anti-PL IgA in saliva. Data are expressed as the arbitrary unit + standard deviation of the mean from three independent experiments. Data from subclasses evaluation are expressed as the OD of samples measured at 492 nm + standard error media (s.e.m) of the means. Significant differences between the means of different groups were determined by a Tukey multiple comparison test using the Graph Pad Prism 4 software (Calif.). A p-value of <0.05 was considered statistically significant.

Figure 2. PL-specific antibody responses induced by SinTimVaS when other mucosal routes were used. Groups of female BALB/c mice ($n = 5$) were SinTimVaS (i.n/i.m, s.l/i.m or o.r/i.m) immunized with AFPL1 (i.m route), AFCo1 (i.n route). Control groups included were i.n, s.l or o.r immunized with three doses of AFCo1 or two i.m doses of AFPL1. The PL-specific IgA in their saliva, vaginal washes and faeces, IgG levels and IgG subtypes in sera were determined using a PL-specific ELISA. (A) Shows the anti-PL IgG levels in sera (B) Shows anti-PL IgG subclasses in sera (C) Shows the anti-PL IgA in saliva, faeces and vagina. Data are expressed as the arbitrary unit + standard deviation of the mean from three independent experiments. Data from subclasses evaluation are expressed as the OD of samples measured at 492 nm + s.e.m of the mean. Significant differences between the means of different groups were determined by a Tukey multiple comparison test using the Graph Pad Prism 4 software (Calif.). Each SinTimVaS combination was compared with 3 doses of the related mucosal route or 2 doses of i.m immunization A p-value of <0.05 was considered statistically significant and it is represented by different letters, a ($p < 0.001$); b is ($p < 0.01$) and c ($p < 0.05$).

Figure 3. PL-specific antibody responses induced by SinTimVaS when subcutaneous priming was used. Groups of female BALB/c mice ($n = 5$) were SinTimVaS (i.n/s.c, s.l/s.c or o.r/s.c) immunized with AFPL1 (s.c route), AFCo1 (i.n route). Control groups included were i.n, s.l or o.r immunized with three doses of AFCo1 or two s.c doses of AFPL1. The PL-specific IgA in their saliva, vaginal washes and faeces, IgG levels and IgG subtypes in sera were determined using a PL-specific ELISA. (A) Shows the anti-PL IgG levels in sera (B) Shows anti-PL IgG subclasses in

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Figure 4. Heterologous specific antibody responses induced by SinTimVaS combinations. Groups of female BALB/c mice ($n = 5$) were SinTimVaS (i.n/i.m, s.l/i.m or o.r/i.m) immunized with BSA and TT antigens using AFPL1 (i.m route) and AFCo1 (i.n route) as adjuvants. Control groups included were i.n, s.l or o.r immunized with three doses of AFCo1 plus BSA or TT or two i.m doses of BSA or TT and AFPL1 adsorbed. The BSA or TT specific IgA in their saliva, IgG levels in sera were determined using a BSA or TT specific ELISA. (A) Shows the anti-BSA IgG levels in sera (B) Shows anti-BSA IgA titer in saliva (C) Shows the anti-TT IgG level in sera (D) Shows anti-TT IgA titer in saliva. Data are expressed as OD of samples measured at 492 nm + s.e.m of the means. Significant differences between the means of different groups were determined by a Tukey multiple comparison test using the Graph Pad Prism 4 software (Calif.). Each SinTimVaS combination was compared with 3 doses of the related mucosal route or 2 doses of i.m immunization A p-value of <0.05 was considered statistically significant and it is represented by different letters, a ($p < 0.001$); b is ($p < 0.01$) and c ($p < 0.05$).

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SinTimVaS combinations

Two priming at the same time, without subsequent boost	
i.n: AFCo1 + BSA / TT	i.m: AFPL1 + BSA / TT
s.l: AFCo1 + BSA / TT	i.m: AFPL1 + BSA / TT
o.r: AFCo1 + BSA /TT	i.m: AFPL1 + BSA / TT

* Adjuvants and antigens concentrations used in SinTimVaS were twice those used in traditional parenteral and mucosal routes

Table 1. Single Time Vaccination Combinations used in the studies.

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