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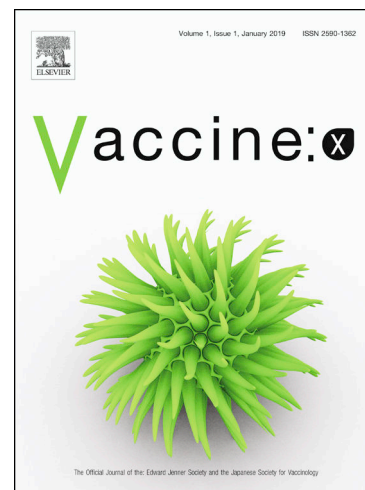
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1 **Crosslinked chitosan microparticles as a safe and efficient DNA carrier for**  
2 **intranasal vaccination against cutaneous leishmaniasis**

3

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33

34 **Abstract**

35

36 Intranasal (i.n.) vaccination with adjuvant-free plasmid DNA encoding the leishmanial  
37 antigen LACK (LACK DNA) has shown to induce protective immunity against both  
38 cutaneous and visceral leishmaniasis in rodents. In the present work, we sought to  
39 evaluate the safety and effectiveness of d,l-glyceraldehyde cross-linked chitosan  
40 microparticles (CCM) as a LACK DNA non-intumescent mucoadhesive delivery system.  
41 CCM with 5  $\mu\text{m}$  of diameter was prepared and adsorbed with a maximum of 2.4% (w/w)  
42 of DNA with no volume alteration. Histological analysis of mouse nostrils instilled with  
43 LACK DNA / CCM showed microparticles to be not only mucoadherent but also  
44 mucopenetrant, inducing no local inflammation. Systemic safeness was confirmed by the  
45 observation that two nasal instillations one week apart did not alter the numbers of  
46 bronchoalveolar cells or blood eosinophils; did not alter ALT, AST and creatinine serum  
47 levels; and did not induce cutaneous hypersensitivity. When challenged in the footpad  
48 with *Leishmania amazonensis*, mice developed significantly lower parasite loads as  
49 compared with animals given naked LACK DNA or CCM alone. That was accompanied  
50 by increased stimulation of Th1-biased responses, as seen by the higher T-bet / GATA-  
51 3 ratio and IFN- $\gamma$  levels. Together, these results demonstrate that CCM is a safe and  
52 effective mucopenetrating carrier that can increase the efficacy of i.n. LACK DNA  
53 vaccination against cutaneous leishmaniasis.

54

55 Key words: Intranasal vaccine, DNA-based vaccine, vaccine delivery systems,  
56 leishmaniasis, chitosan microparticles. Glyceraldehyde crosslinking

57

58

## 59 Introduction

60

61 Leishmaniasis is a vector borne neglected disease caused by intracellular  
62 protozoan parasites of the genus *Leishmania*. It may display a wide spectrum of clinical  
63 manifestations ranging from benign cutaneous lesions to fatal visceral infection. Around  
64 600,000 to 1 million new cases of cutaneous leishmaniasis (CL) and 50,000 to 90,000  
65 cases of visceral leishmaniasis (VL) are estimated to occur in 98 countries worldwide [1].  
66 Despite the recent advances in the discovery of new antileishmanial compounds,  
67 chemotherapy remains inadequate [2]. Although vaccination is considered the most  
68 effective means for disease prevention, there is as yet no vaccine approved for human  
69 leishmaniasis [3].

70 A successful leishmanial infection relies on the parasite ability to rapidly induce  
71 Th2-type immune responses that in turn prevent activation of effective Th1 and other  
72 pro-inflammatory responses involved in parasite killing [4]. We and others have  
73 hypothesized that prevention of the robust Th2 responses can be a more effective way  
74 to allow the peripheral expansion of protective Th1 responses than the classical  
75 vaccination with weaker Th1-biasing antigens [5]. That assumption is supported by the  
76 early study by Julia V *et. al* (1996) showing that mice rendered tolerant to the strong Th2-  
77 biasing antigen LACK (*Leishmania* homologue of receptors for activated C kinase) are  
78 more resistant to *L. major* infection than wild-type animals [6]. As an alternative to thymic  
79 expression from birth as used in that study, we have successfully achieved tolerance to  
80 LACK through mucosal vaccination. LACK DNA given by the intranasal (i.n.) route have  
81 induced protection in mice and hamsters against both cutaneous and visceral  
82 leishmaniasis [7, 8, 9, 10, 11, 12, 13, 14, 15, 16]. The superiority of the i.n. route in  
83 relation to the classical subcutaneous (s.c.) and intramuscular routes was demonstrated  
84 with both LACK-containing whole *Leishmania amazonensis* lysate (LaAg) and LACK  
85 DNA itself [7, 13, 15, 17, 18, 19].

86 However, nasal mucus may reduce antigen contact and uptake by the nasal  
87 epithelium and reduce sensitization of the mucosal immune system. Polymeric  
88 nanoparticles and microparticles have been extensively explored as effective drug  
89 carriers [20]. Chitosan particles are particularly interesting due to their strong positive  
90 charge that enables binding to mucins and mucoadhesiveness, besides electrostatic  
91 interaction with negatively charged DNA. Their strong cationic nature can also promote  
92 opening of intercellular tight junctions and penetration through the mucosal epithelium  
93 [21, 22, 23]. Since chitosan particles are highly hygroscopic, polymer crosslinking is  
94 important to avoid hydrogel formation and nasal obstruction. Crosslinking with aldehydes  
95 such as glutaraldehyde and formaldehyde can be very effective, but their toxicity limits  
96 pharmaceutical utilization. On the other hand, as a metabolic product of fructose, d,l-  
97 glyceraldehyde is expectedly non-toxic and more adequate for mucosal administration  
98 [24].

99 In the present study, we aimed to investigate the d,l-glyceraldehyde-crosslinked  
100 chitosan microparticle (CCM) biocompatibility with the nasal mucosa, and its capacity to  
101 maximize the protective effect of i.n. LACK DNA vaccine against cutaneous  
102 leishmaniasis.

103

## 104 Materials and Methods

105

106 *Animals and ethics statement:* BALB/c mice (male, eight-week-old) were maintained  
107 under controlled conditions of room temperature, autoclaved bedding, pelleted food and  
108 filtered water. All animal experiments in this study were conducted in compliance with  
109 the principles stated in the Guide for the Care and Use of Laboratory Animals (8th  
110 Edition, 2011), pre-approved by the Animal Care and Use Committee of the Federal  
111 University of Rio de Janeiro (Brazil) under the code CAUAP118.

112

113 *d,l-glyceraldehyde cross-linked chitosan microparticles (CCM):* Chitosan microparticles  
114 were prepared by spray drying technique as adapted from Oliveira et al. [24]. Briefly, a  
115 solution of 0.7% w/v acetic acid (Sigma-Aldrich) containing 2.5 % of chitosan of medium  
116 molecular weight (Sigma-Aldrich) and subjected to spray drying (Büchi, Switzerland). For  
117 cross-linking, particles were suspended in an acetone:water solution (2:1) containing  
118 1.5% of d,l-glyceraldehyde under agitation at 500 rpm for 30 min at room temperature.  
119 Then, CCM were dried under vacuum at room temperature for 24 h and stocked under  
120 desiccation. Particle size distribution was measured by laser diffraction technique  
121 (MasterSizer model E, Malvern, UK), while the zeta potential was measured using a  
122 Zetamaster (Malvern, UK). For scanning electron microscopy (SEM), dry microparticles  
123 were sputtered on double-face tape and these with gold (JFC-129 1300, JEOL) prior to  
124 analysis in electron microscope (JPC 1100, JEOL) at 15 kV [25].

125

126 *LACK DNA adsorption to CCM (LACK DNA/CCM):* The pCI-neo plasmid with p36LACK  
127 insert (LACK DNA) was used to transform *Escherichia coli* DH-5 $\alpha$ <sup>TM</sup> (Invitrogen). LACK  
128 DNA was extracted from *E. coli* cultures by the alkaline lysis method using a DNA  
129 lipopolysaccharide (LPS)-free extraction kit (Qiagen Giga-Prep - USA) according to  
130 manufacturer's instructions. For adsorption onto CCM, varying amounts of LACK DNA  
131 (0.2 mg, 0.4 mg, 0.6 mg, 0.8 mg, 1 mg, 1.2 mg, 1.4 mg, 1.6 mg, 1.8 mg and 2 mg) were  
132 mixed with 50 mg of CCM in 25 mL solution of citrate-phosphate buffer and ethanol (2:1)  
133 pH 5.5. The suspension was incubated for 2 h at 37 °C under agitation of 100 rpm. After  
134 this time, samples were washed two times with 10 mL of citrate-phosphate /ethanol  
135 buffer above at 1500 rpm / 10 min, and the rate of adsorbed LACK DNA was determined  
136 in triplicate in a spectrophotometer (NanoDrop 2000, Thermo Scientific) at 260 nm.  
137 Adsorption was calculated by subtracting the amount of DNA in the supernatant from  
138 added DNA at the start of incubation. After adsorption, LACK DNA/CCM (1.2 mg:50 mg)  
139 was resuspended in PBS to 1 mg of CCM/mL One hundred microliters of that suspension  
140 were labeled with 125  $\mu$ M of ethidium bromide (EtBr) (Sigma-Aldrich). CCM was treated  
141 with EtBr in the same way. Images were obtained under visible and ultraviolet lamp in a  
142 fluorescence microscope (AxioVision).

143

144 *Allergenicity:* Mice were given two i.n. doses with 2 mg of CCM in 20  $\mu$ L of PBS (10  $\mu$ L  
145 in each nostril) 7 days apart. Controls received 50  $\mu$ g of s.c. ovalbumin (OVA) (Sigma-  
146 Aldrich) in the same days. For early and late cutaneous hypersensitivity, one week after  
147 the last dose animals were challenged s.c. in the footpad with 50  $\mu$ g of CCM or 50  $\mu$ g of  
148 OVA in 20  $\mu$ L of PBS, and footpad swelling was measured with a dial caliper 2 h and 24  
149 h later. For eosinophilia, 24 h after challenge mouse blood was collected and eosinophils  
150 were counted in Fast Panoptic (Instant-Prov, Newprov) - stained blood smears and  
151 expressed in relation to total leukocytes (> 400 cells/ sample). For IgE, 24 h after  
152 challenge this was assessed in the mouse serum by ELISA using a mouse IgE ELISA  
153 kit (BD Bioscience) according to the manufacturer's instructions.



154

155 *Acute systemic toxicity*: we developed protocols aimed to evaluate the systemic  
156 biocompatibility of CCM, based on "Non-Clinical Safety Testing" (TDR/WHO, 2004).  
157 Mice were given two i.n. doses with 2 mg of CCM in 20  $\mu$ L of PBS seven days apart. For  
158 liver and kidney toxicity, positive controls received a single intraperitoneal (i.p.) injection  
159 with 1% carbon tetrachloride ( $\text{CCl}_4$ , Sigma-Aldrich) in 100  $\mu$ L of peanut oil. Mice were  
160 euthanized 24 h after the last sensitizing dose, and aspartate aminotransferase (AST),  
161 alanine aminotransferase (ALT) and creatinine were determined at the serum by  
162 colorimetric laboratory diagnostic kits according to the manufacturer's instructions  
163 (Dolles, Brazil). Clinical signs of acute toxicity were evaluated twice a day for 7 days. For  
164 lung inflammation animals received two i.n. doses of CCM or PBS as above. Positive  
165 controls received two i.n. doses with 10  $\mu$ g of LPS in 20  $\mu$ L of PBS. Mice were euthanized  
166 2 h after the second dose, the bronchoalveolar lavage (BAL) fluid collected in 1 mL of  
167 PBS, and the total number of cells were counted using a Neubauer chamber.

168

169 *Histology of nasal mucosa*: Mice received one dose with 30  $\mu$ g of LACK DNA/1.2 mg  
170 CCM in 20  $\mu$ L of PBS by i.n. route. Fifteen minutes after the administration, mice were  
171 euthanized, their whole heads were fixed for one week in 10% formaldehyde solution  
172 and decalcified during further 2 weeks using a 14% ethylenediaminetetraacetic acid  
173 (EDTA) (Sigma, USA) solution. Then, they were dehydrated, embedded in paraffin and  
174 sliced at a thickness of 4  $\mu$ m. The slices were deparaffinized and stained with Masson's  
175 trichrome.

176

177 *Vaccination and infection*: Mice were given two i.n. doses seven days apart with 30  $\mu$ g  
178 of naked LACK DNA; 30 $\mu$ g LACK DNA/1.2mg CCM (LACK DNA / CCM); or 1.2 mg of  
179 naked CCM in 20  $\mu$ L of PBS (10  $\mu$ L in each nostril). Controls received phosphate buffer  
180 solution (PBS) alone or naked CCM in equivalent amounts to LACK DNA. One week  
181 after the second dose, animals were s.c. injected in the hind footpad with  $10^5$   
182 promastigotes of *L. amazonensis* (MHOM/BR/75/Josefa). The lesion growth was  
183 monitored by periodical measuring the thickness of the footpads with a dial caliper. On  
184 day 107 of infection (day 121 post vaccination), mice were euthanized and the parasite  
185 loads in the infected footpads were determined by limiting-dilution assay (LDA), as  
186 previously described [26]. Briefly, the feet were excised at the knee junction, aseptically  
187 skinned, and individually homogenized in M199 medium supplemented with antibiotics  
188 (50  $\mu$ g/mL of streptomycin plus 50 U/mL of penicillin) and 20% heat inactivated fetal calf  
189 serum (Cultilab, Brazil). Triplicate serial dilutions were made in 100  $\mu$ L of the same  
190 medium in flat bottom microplates that were incubated at 26°C in humid chambers for 10  
191 to 14 days. The original numbers of parasites in each footpad was calculated from the  
192 reciprocal of the highest dilution containing promastigotes.

193

194 *Reverse transcription quantitative PCR (RT-qPCR)*: Three days after infection challenge,  
195 lesion-draining popliteal lymph nodes were homogenized in guanidine isothiocyanate  
196 solution 4 M. Complementary DNA (cDNA) was synthesized through ImProm-IITM  
197 Reverse Transcription System (Promega, USA) according to manufacturer instructions.  
198 RT-qPCR reactions were performed in triplicates for each target in StepOne system  
199 (Applied Biosystems, USA) using StepOne v2.1 software. Each reaction consisted of  
200 yielded cDNA, 2x SYBER Green Master Mix (Applied Biosystems, USA), forward primer  
201 (F) and reverse primer (R) (5 mM each, Promega Corporation, USA). Primers were

202 designed using Primer Express software version 3.0 (Applied Biosystems, USA):  
203 Foxp3F: 5' -GGC CCT TCT CCA GGA CAG A-3', Foxp3R: 5' -GGC ATG GGC ATC  
204 CAC AGT-3' , GATA3F: 5' -GAC CCG AAA CCG GAA GAT GT-3' , GATA3R: 5' -GCG  
205 CGT CAT GCA CCT TTT3' , TbetF:5' - GCC AGG GAA CCG CTT ATA TG-3' , TbetR:  
206 5' -AAC TTC CTG GCG CAT CCA-3'. RT-qPCR reaction conditions were: 40 cycles of  
207 95° C for 30 sec and 60°C for 1 min. The gene expression of GAPDH was used as  
208 endogenous control: GAPDHF 5' -GGG AAG CCC ATC ACC ATC T-3', GAPDHR 5' -  
209 GCC TCA CCC CAT TTG ATG TT-3'.

210

211 *Cytokine production:* Infected feet were individually homogenized, and centrifuged at  
212 2000g/10 min for clarification. IFN- $\gamma$  was quantitated by ELISA using paired rat IgG1 anti-  
213 mouse IFN- $\gamma$  (clone XMG1.2) and biotinylated rat IgG1 anti-mouse IFN- $\gamma$  (clone R4-6A2)  
214 antibodies. Standard curves were prepared with recombinant cytokines according to  
215 manufacturer instructions (R&D Systems, USA).

216

217 *Statistical Analysis:* Statistical analysis was employed using One-Way ANOVA and  
218 Dunnett post-test for comparison between different groups. These were considered  
219 significantly different when  $p < 0.05$ .

220

221

## 222 **Results**

223

### 224 **Microparticle physical properties**

225 Size distribution and zeta potential were determined in CCM showing a mean diameter  
226 of 4.77  $\mu\text{m}$  at D (4.3) with an acceptable 2.21 span value, and surface charge (zeta  
227 potential) of  $+29.8 \pm 1.0$  mV, compatible with cationic property of chitosan. Adsorption  
228 of LACK DNA to CCM reduced the zeta potential to  $+20.8 \pm 0.6$  likely due to the anionic  
229 nature of DNA, while the mean particle size remained unchanged (data not shown).

230

### 231 **Absence of i.n. CCM allergenicity**

232 The safety of CCM was first evaluated according to its capacity to induce topical  
233 allergy. For that, animals were pre-sensitized with two i.n. doses of 2 mg of CCM or two  
234 s.c. doses with 50  $\mu\text{g}$  of the known allergen OVA, followed by a s.c. challenge in the  
235 footpad with the homologous stimuli one week later. Unlike pre-sensitization with OVA  
236 that led to significant footpad swelling 2 and 24 h after homologous challenge, i.n. CCM  
237 did not lead to increased swelling in comparison to PBS controls (Figure 1A). The basal  
238 CCM response was more prominent after 24 h than after 2 h probably due to injection  
239 itself. Of note, the intended use of CCM is topical in the mucosa not injected s.c., and  
240 that animals did not show discomfort after nasal instillation, such as sniffing or scratching  
241 as monitored up to 30 h of application. Absence of allergenicity with i.n. CCM, and even  
242 s.c. CCM, is confirmed by absence of increased eosinophilia as compared with s.c. OVA  
243 (Figure 1B) or total IgE production (Figure 1C). Together, these data indicate that in the  
244 tested conditions CCM neither induces local nor systemic allergic reactions.



245

**246 I.n. CCM does not induce detectable systemic toxicity**

247 To evaluate the general safety of CCM as a nasal carrier, the serum levels of  
248 AST, ALT and creatinine were measured 24 h after the second i.n. dose. Figures 2A-C  
249 show that a total of 4 mg of CCM given intranasally did not induce detectable alterations  
250 in any of the biochemical markers, as compared to controls receiving PBS alone.

251 The capacity of i.n CCM to induce lung inflammation was also investigated. For  
252 that, the BAL was used to monitor lower airway cell infiltration due both to the difficulty  
253 in performing nasal lavage, and the possibility that the nasal particles reach the lungs.  
254 Two hours after the second dose of CCM, the BAL was collected, and total leukocytes  
255 counted. Unlike instillation with LPS that led to increased BAL cellularity, CCM did not  
256 affect cell counts as compared with PBS controls (Figure 2D). Together, these results  
257 show that the glyceraldehyde crosslinked particles are biocompatible and do not induce  
258 detectable toxicity to the heart, liver, kidneys and lungs.

259

**260 CCM is an efficient LACK DNA carrier system**

261 After confirmation of biocompatibility, CCM were tested for the capacity to adsorb  
262 and serve as delivery systems for i.n. DNA vaccines. After incubation of CCM with  
263 varying amounts of LACK at pH 5.5 for 2 h, DNA was quantified in the supernatant.  
264 Figure 3A shows that as much as 1.2 mg of DNA can be 100% adsorbed onto 50 mg of  
265 CCM, i.e., 0.6% loading capacity (w/w). Addition of higher LACK DNA amounts results  
266 in decreased loading efficiency.

267 To demonstrate LACK DNA adsorption to CCM, ethidium bromide was added to  
268 the complex, particles were washed and observed under a fluorescence microscopy  
269 (Figure 4B). Unlike naked CCM that showed no fluorescence of ethidium bromide when  
270 exposed to UV light, LACK DNA/CCM showed a high red fluorescence. LACK DNA was  
271 stably adsorbed, as no DNA was quantifiable in the supernatants for at least 72 hours  
272 after suspension in PBS (not shown). These results also show that the glyceraldehyde  
273 used for chitosan crosslinking did not prevent the electrostatic binding of DNA, stable  
274 enough to resist washing with buffer at pH 5.5, like that of nasal lumen.

275

**276 CCM is an efficient LACK DNA mucosal delivery system**

277 To assess the ability of CCM to deliver LACK DNA at the nasal mucosa, the  
278 adhesion and interaction of LACK DNA/CCM with the epithelium was investigated *in vivo*.  
279 After 15 min of i.n. instillation with 1.2 mg of LACK DNA / CCM in 20  $\mu$ L of PBS (10  $\mu$ L  
280 in each nostril), mice were euthanized and microparticle interaction with the nasal  
281 epithelium was assessed in histological sections stained with Masson's trichrome. Figure  
282 4A shows a mouse nose section with sinuses and central septum with the localization of  
283 inlets B and C, magnified in Figures 4B and 4C, respectively. Red LACK DNA/CCM  
284 microparticles are clearly seen adhering and traversing the mucosal epithelium within  
285 only 15 min, demonstrating their rapid mucoadhesiveness and penetrating ability.

286

**287 Efficacy of LACK DNA/CCM as mucosal vaccine against leishmaniasis**

288 We have previously demonstrated the partial efficacy of naked LACK DNA i.n.  
289 vaccination against CL (*L. amazonensis*) [7] and VL (*L. infantum*) [9, 10]. To investigate  
290 whether the strong mucoadhesiveness and penetrating ability of LACK DNA/CCM could  
291 enhance vaccine efficacy, mice were pre-vaccinated here with LACK DNA / CCM and  
292 naked LACK DNA as in those studies. Controls received naked CCM or PBS vehicle in  
293 equivalent amounts. One week after the boost dose animals were subcutaneously  
294 challenged in the footpad with *L. amazonensis*. Vaccine efficacy was monitored both by  
295 the growth of lesions through infection and parasite load at the end of the experiment.  
296 Figure 5A shows that pre-vaccination with LACK DNA/CCM significantly ( $p<0.01$ )  
297 delayed lesion growth as compared with naked DNA, which at a lesser extent was also  
298 delayed ( $p<0.05$ ) in relation to PBS controls. Naked CCM was inert. Consistent with  
299 their slower lesion growth, animals that were pre-vaccinated with LACK DNA/CCM  
300 developed significantly ( $p<0.001$ ) lower parasite burdens than non-vaccinated controls  
301 and animals given naked LACK DNA (Figure 5B).

302

### 303 **LACK DNA/CCM vaccination promoted Th1-type immune responses**

304 The possibility that vaccination efficacy was associated with skewed Th1 responses was  
305 investigated. Indeed, increased T-bet concomitant with decreased GATA-3 and Foxp3  
306 gene expression was observed in the lesion-draining lymph nodes of vaccinated mice,  
307 as compared with non-vaccinated mice in the critical early days of infection (day 3, Figure  
308 6 A) measured three days after challenge Early Th1 polarization resulted in long lasting  
309 capacity of LACK DNA/CCM to produce IFN- $\gamma$  in comparison to naked LACK DNA (day  
310 107, Figure 6 B). These results indicate that in some way, LACK DNA / CCM may be  
311 more effective than naked LACK DNA in stimulating a protective immunity.

312

### 313 **Discussion**

314

315 In the last two decades, micro- and nanoparticles of chitosan have been  
316 described as interesting carriers for gene delivery [27]. They can increase mucosal DNA  
317 delivery by protecting plasmids from the action of DNases in the mucosa; by adhering to  
318 the mucosa and slowly releasing the DNA, increasing its absorption; and /or improving  
319 the capture of particulate antigens by microfold cells (M cells) and, subsequently, by  
320 antigen-presenting cells in the lamina propria [28]. In this work, we attempted to improve  
321 the efficacy of LACK DNA vaccine by adsorption onto CCM, and attest its safety to the  
322 nasal mucosa.

323 The extend of *d,l*-glyceraldehyde cross-linking might determine the number of  
324 free amino groups and the positively charged surface of CCM [29]. We observed that our  
325 crosslinking process using 10-fold less *d,l*-glyceraldehyde than used previously [24] did  
326 not interfere with the ability of chitosan to bind LACK DNA. An adsorption rate of 100%  
327 was achieved by using 1.2 mg of LACK DNA and 50 mg of CCM, as compared with other  
328 studies where only 78% and 38% of plasmid DNA adsorption was obtained using similar  
329 ratios of plasmid DNA with CCM [30], or polyethylene glycol microparticles [31],  
330 respectively.

331 The histological analysis showed that LACK DNA/CCM attached and entered the  
332 nasal mucosal epithelium probably by interactions with the negatively charged mucus  
333 mucins [32] followed either by transportation by M cells [33] or paracellular route through

334 tight junctions [34, 35]. No sign of local irritancy or cell infiltration was observed with CCM  
335 as opposing to the traditional crosslinking agent glutaraldehyde that is highly cytotoxic  
336 [36].

337 Also, no changes in systemic toxicity parameters were observed, as serum levels  
338 of AST, ALT and creatinine and bronchoalveolar cell infiltration remained unchanged  
339 after simulation of vaccination schedule with CCM. There is a concern regarding the use  
340 of chitosan in individuals allergic to shellfish, since it is obtained through the  
341 deacetylation of chitin present in the crustacean exoskeleton. However, topical chitosan  
342 bandages proved to be safe in allergic patients [37]. Likewise, no cutaneous sensitization  
343 was induced in mice following repeated nasal administration of CCM.

344 Although intranasal vaccination with LACK DNA alone result in skewed Th1-type  
345 immune response in the lesion-draining lymph nodes, this can be pronounced with LACK  
346 DNA /CCM. CCM mucosal adjuvanticity apparently operates in a way different from solid  
347 lipid nanoparticles (SLN) loaded with retinoic acid that were used to improve the nasal  
348 efficacy of LaAg. In that case, the adjuvant effect of SLN was associated with increased  
349 Treg cells at the site of infection [38].

350 This work demonstrated that CCM can be used as a safe i.n. delivery system for  
351 LACK DNA vaccine against leishmaniasis. The potential clinical use of CCM as a safe  
352 mucoadhesive DNA carrier vaccine for leishmaniasis and other diseases is  
353 strengthened by the recent licensing by the government of India of the world's first  
354 DNA-based for use in people aged 12 years or older against COVID-19, the ZyCov-D  
355 [39]. According to the World Health Organization's 'COVID-19 Vaccine Tracker and  
356 Landscape', as per today 15 other DNA vaccines, 8 i.n. and 4 oral vaccines are also in  
357 clinical development against the novel coronavirus, which may benefit from CCM as a  
358 safe mucosal DNA carrier described here.

359

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363

## 364 References

365

366 1. Carrion C, Robles N, Sola-Morales O, Aymerich M, Ruiz Postigo, JA. Mobile health  
367 strategies to tackle skin neglected tropical diseases with recommendations from  
368 innovative experiences: Systematic review. JMIR Mhealth Uhealth 2020; 8(12):e22478.  
369 doi: 10.2196/22478.

370

371 2. Ponte-Sucre A, Gamarro F, Dujardin JC, Barrett MP, López-Vélez R, García-  
372 Hernández R, *et al.* Drug resistance and treatment failure in leishmaniasis: A 21st  
373 century challenge. PLoS Negl Trop Dis 2017; 11(12):e0006052. doi:  
374 10.1371/journal.pntd.0006052.

375

- 376 3. Iborra S, Solana JC, Requena JM, Soto M. Vaccine candidates against leishmania  
377 under current research. *Expert Rev Vaccines* 2018; 17(4):323-334. doi:  
378 10.1080/14760584.2018.1459191.
- 379
- 380 4. Carneiro, M.B., Lopes, M.E., Hohman, L.S., Romano, A., David, B.A., Kratofil, R.,  
381 Kubes, P., Workentine, M.L., Campos, A.C., Vieira, L.Q., and Peters, N.C. (2020). Th1-  
382 Th2 Cross-Regulation Controls Early Leishmania Infection in the Skin by Modulating the  
383 Size of the Permissive Monocytic Host Cell Reservoir. *Cell Host Microbe* 27, 752-768.e7.  
384 doi:10.1016/j.chom.2020.03.011
- 385
- 386 5. Campos-Neto A. What about Th1/Th2 in cutaneous leishmaniasis vaccine discovery?  
387 *Braz J Med Biol Res.* 2005 Jul;38(7):979-84. doi: 10.1590/s0100-879x2005000700001.
- 388
- 389 6. Julia V, Rassoulzadegan M, Glaichenhaus N. Resistance to *Leishmania major*  
390 induced by tolerance to a single antigen. *Science* 1996; 274(5286):421-3. doi:  
391 10.1126/science.274.5286.421.
- 392
- 393 7. Pinto EF, Pinheiro RO, Rayol A, Larraga V, Rossi-Bergmann B. Intranasal vaccination  
394 against cutaneous leishmaniasis with a particulated leishmanial antigen or DNA  
395 encoding LACK. *Infection and Immunity* 2004;4521–4527. doi: 10.1128/IAI.72.8.4521-  
396 4527.2004.
- 397
- 398 8. Pinheiro RO, Pinto EF, de Matos Guedes HL, Filho OA, de Mattos KA, Saraiva EM,  
399 *et al.* Protection against cutaneous leishmaniasis by intranasal vaccination with  
400 lipophosphoglycan. *Vaccine* 2007; 25(14):2716-22. doi: 10.1016/j.vaccine.2006.05.093.
- 401
- 402 9. Gomes DCO, Pinto EF, Melo LDB, Lima WP, Larraga V, Lopes UG, *et al.* Intranasal  
403 delivery of naked DNA encoding the LACK antigen leads to protective immunity against  
404 visceral leishmaniasis in mice. *Vaccine* 2007; 25:2168–2172. doi:  
405 10.1016/j.vaccine.2006.11.060.
- 406
- 407 10. Gomes DCO, Souza BLSC, de Matos Guedes HL, Lopes UG, Rossi-Bergmann, B.  
408 Intranasal immunization with LACK-DNA promotes protective immunity in hamsters  
409 challenged with *Leishmania chagasi*. *Parasitology* 2011;138(14):1892-7.  
410 doi: 10.1017/S0031182011001417.
- 411
- 412 11. de Matos Guedes HL, da Silva Costa BL, Chaves SP, de Oliveira Gomes DC,  
413 Nosanchuk JD, De Simone SG, *et al.* Intranasal vaccination with extracellular serine

414 proteases of *Leishmania amazonensis* confers protective immunity to BALB/c mice  
415 against infection. *Parasit Vectors* 2014; 7:448. doi: 10.1186/1756-3305-7-448.

416

417 12. Leal JM, Mosquini M, Covre LP, Stagmiller NP, Rodrigues RR, Christensen D, *et al.*  
418 Intranasal vaccination with killed *Leishmania amazonensis* promastigotes antigen (LaAg)  
419 associated with CAF01 adjuvant induces partial protection in BALB/c mice challenged  
420 with *Leishmania (infantum) chagasi*. *Parasitology* 2015; 142(13):1640-6. doi:  
421 10.1017/S0031182015001250.

422

423 13. Da Silva-Couto L, Ribeiro-Romão RP, Saavedra AF, Costa BLS., Moreira OC,  
424 Gomes-Silva A, *et al.* Intranasal vaccination with leishmanial antigen protects golden  
425 hamster (*Mesocricetus auratus*) against *Leishmania (Viannia) braziliensis* infection.  
426 *PLoS Neglected Tropical Diseases* 2015; 9(1):e3439. doi:  
427 10.1371/journal.pntd.0003439.

428

429 14. Pratti JE, Ramos TD, Pereira JC, da Fonseca-Martins AM, Maciel-Oliveira D,  
430 Oliveira-Silva G, *et al.* Efficacy of intranasal LaAg vaccine against *Leishmania*  
431 *amazonensis* infection in partially resistant C57Bl/6 mice. *Parasit Vectors* 2016;  
432 9(1):534. doi: 10.1186/s13071-016-1822-9.

433

434 15. Pereira Silva Bezerra I, Amaral Abib M, Rossi-Bergmann B. Intranasal but not  
435 subcutaneous vaccination with LaAg allows rapid expansion of protective immunity  
436 against cutaneous leishmaniasis. *Vaccine* 2018; 36(18):2480-2486. doi:  
437 10.1016/j.vaccine.2018.03.020.

438

439 16. Pratti JES, da Fonseca Martins AM, da Silva JP, Ramos TD, Pereira JC, Firmino-  
440 Cruz L, *et al.* The role of TLR9 on *Leishmania amazonensis* infection and its influence  
441 on intranasal LaAg vaccine efficacy. *PLoS Negl Trop Dis* 2019; 13(2):e0007146. doi:  
442 10.1371/journal.pntd.0007146.

443

444 17. Pinto EF, de Mello Cortezia M, Rossi-Bergmann B. Interferon-gamma-inducing oral  
445 vaccination with *Leishmania amazonensis* antigens protects BALB/c and C57BL/6 mice  
446 against cutaneous leishmaniasis. *Vaccine* 2003; 21(25-26):3534-41. doi:  
447 10.1016/s0264-410x(03)00427-4.

448

449 18. Pinheiro RO, Pinto EF, Lopes JR, Guedes HL, Fentanes RF, Rossi-Bergmann B.  
450 TGF-beta-associated enhanced susceptibility to leishmaniasis following intramuscular  
451 vaccination of mice with *Leishmania amazonensis* antigens. *Microbes Infect* 2005;  
452 7(13):1317-23. doi: 10.1016/j.micinf.2005.04.016.

453



- 454 19. Marques-da-Silva EA, Coelho EA, Gomes DC, Vilela MC, Masioli CZ, Tavares CA *et*  
455 *al.* Intramuscular immunization with p36(LACK) DNA vaccine induces IFN-gamma  
456 production but does not protect BALB/c mice against *Leishmania chagasi* intravenous  
457 challenge. *Parasitol Res* 2005; 98(1):67-74. doi: 10.1007/s00436-005-0008-8.
- 458
- 459 20. Bookstaver ML, Tsai SJ, Bromberg JS, Jewell CM. Improving Vaccine and  
460 Immunotherapy Design Using Biomaterials. *Trends Immunol* 2018; 39(2):135-150. doi:  
461 10.1016/j.it.2017.10.002.
- 462
- 463 21. Buschmann MD, Merzouki A, Lavertu M, Thibault M, Jean M, Darras V. Chitosans  
464 for delivery of nucleic acids. *Adv Drug Deliv Rev* 2013; 65(9):1234-70. doi:  
465 10.1016/j.addr.2013.07.005.
- 466
- 467 22. Kumar A, Vimal A, Kumar A. Why Chitosan? From properties to perspective of  
468 mucosal drug delivery. *Int J Biol Macromol* 2016; 91:615-22. doi:  
469 10.1016/j.ijbiomac.2016.05.054.
- 470
- 471 23. Kravanja G, Primožič M, Knez Ž, Leitgeb M. Chitosan-based (Nano)materials for  
472 Novel Biomedical Applications. *Molecules* 2019; 24(10):1960. doi:  
473 10.3390/molecules24101960.
- 474
- 475 24. Oliveira BF, Santana MHA, Ré MI. Spray-dried chitosan microspheres cross-linked  
476 with d,l-glyceraldehyde as a potential drug delivery system: preparation and  
477 characterization. *Brazilian Journal of Chemical Engineering* 2005; 22(03):353-360.  
478 doi: 10.1590/S0104-66322005000300004
- 479
- 480 25. Sousa-Batista AJ, Pacienza-Lima W, Ré MI, Rossi-Bergmann B. Novel and safe  
481 single-dose treatment of cutaneous leishmaniasis with implantable amphotericin B-  
482 loaded microparticles. *Int J Parasitol Drugs Drug Resist.* 2019;11:148-155. doi:  
483 10.1016/j.ijpddr.2019.06.001.
- 484
- 485 26. Titus RG, Marchand M, Boon T, Louis JA. A limiting dilution assay for quantifying  
486 *Leishmania major* in tissues of infected mice. *Parasite Immunol* 1985; 7(5):545-55. doi:  
487 10.1111/j.1365-3024.1985.tb00098.x.
- 488
- 489 27. Cao Y, Tan YF, Wong YS, Liew MWJ, Venkatraman S. Recent advances in chitosan-  
490 based carriers for gene delivery. *Mar Drugs* 2019; 17(6):381. doi:10.3390/md17060381
- 491



- 492 28. Mao S, Sun W, Kissel T. Chitosan-based formulations for delivery of DNA and siRNA.  
493 Adv Drug Deliv Rev 2010; 62(1):12-27. doi: 10.1016/j.addr.2009.08.004.  
494
- 495 29. Devraj KM, Ramanjaneyulu K, Hima Bindu N, Ramakrishnamacharya SV.  
496 Preparation and Characterization of Chitosan Microspheres Crossed-linked with 2, 3 -  
497 Dihydroxypropanal as a competitive Drug Delivery System. J Pharmacy Research 2011;  
498 4(5),1433-1435. doi: 10.1081/ddc-120018377  
499
- 500 30. Oliveira BF, Santana MHA, Ré MI. Spray-Dried Chitosan Microspheres as a pDNA  
501 Carrier. Drying Technology 2006; 24: 373-382.  
502
- 503 31. Chen Z, Cai X, Yang Y, Wu G, Liu Y, Chen F. *et al.* Promoted transfection efficiency  
504 of pDNA polyplexes-loaded biodegradable microparticles containing acid-labile  
505 segments and galactose grafts. Pharmaceutical Research 2012; 2:471-82. doi:  
506 10.1007/s11095-011-0577-4.  
507
- 508 32. Dhawan S, Singla AK, Sinha VR. Evaluation of mucoadhesive properties of chitosan  
509 microspheres prepared by different methods. AAPS Pharm Sci Tech 2004; 5(4):e67. doi:  
510 10.1208/pt050467.  
511
- 512 33. Kyd JM, Cripps AW. Functional differences between M cells and enterocytes in  
513 sampling luminal antigens. Vaccine 2008; 26(49): 6221-4. doi:  
514 10.1016/j.vaccine.2008.09.061.  
515
- 516 34. Dodane V, Amin Khan M, Merwin JR. Effect of chitosan on epithelial permeability  
517 and structure. Int J Pharm 1999; 182(1):21-32. doi: 10.1016/s0378-5173(99)00030-7.  
518
- 519 35. Vllasaliu D, Exposito-Harris R, Heras A, Casettari A, Garnett M. *et al.* Tight junction  
520 modulation by chitosan nanoparticles: comparison with chitosan solution. Int J Pharm  
521 2010; 400(1-2):183-93. doi: 10.1016/j.ijpharm.2010.08.020.  
522
- 523 36. Nair, M.; Best, S.M.; Cameron, R.E. Crosslinking collagen constructs: Achieving  
524 cellular selectivity through modifications of physical and chemical properties. Appl.  
525 Sci. 2020; 10:6911-. <https://doi.org/10.3390/app10196911>.  
526
- 527 37. Waibel KH, Haney B, Moore M, Whisman B, Gomez R. Safety of chitosan bandages  
528 in shellfish allergic patients. Mil Med 2011; 176(10):1153-6. doi: 10.7205/milmed-d-11-  
529 00150.

530

531 38. Bezerra IPS, Costa-Souza BLS, Carneiro G, Ferreira LAM, de Matos Guedes HL,  
532 Rossi-Bergmann B. Nanoencapsulated retinoic acid as a safe tolerogenic adjuvant for  
533 intranasal vaccination against cutaneous leishmaniasis. *Vaccine*. 2019 Jun 19;  
534 37(28):3660-3667. doi: 10.1016/j.vaccine.2019.05.043.

535

536 39. Momin T, Kansagra K, Patel H, Sharma S, Sharma B, Patel J, Mittal R, Sanmukhani  
537 J, Maithal K, Dey A, Chandra H, Rajanathan CT, Pericherla HP, Kumar P, Narkhede A,  
538 Parmar D. Safety and Immunogenicity of a DNA SARS-CoV-2 vaccine (ZyCoV-D):  
539 Results of an open-label, non-randomized phase I part of phase I/II clinical study by  
540 intradermal route in healthy subjects in India. *EClinicalMedicine*. 2021 38:101020. doi:  
541 10.1016/j.eclinm.2021.101020.

542

## 543 Figures

544

545 **Figure 1 - Allergenicity of CCM.** Mice were immunized with two i.n. doses of CCM (2  
546 mg) with a 7-day interval. Controls were given s.c. OVA or s.c. PBS. One week later,  
547 animals were challenged with CCM or OVA. A) Footpad swelling was measured with a  
548 caliper 2 and 24 h after challenge. B) Twenty four hours after challenge, the number of  
549 blood eosinophils was counted in the blood and C) IgE production was assessed in the  
550 mouse serum by ELISA. Arithmetic means  $\pm$  SD (n = 3) \*\*\* p<0.01 as compared to PBS.

551

552 **Figure 2. Acute toxicity of CCM.** Mice received two i.n. doses of CCM (2 mg), PBS (20  
553  $\mu$ L), or LPS (10  $\mu$ L) with a 7-day interval, or a single i.p. injection with 1 % CCl<sub>4</sub>, as  
554 indicated. A-C) Twenty four hours after the second dose, the serum was collected for  
555 determination of the indicated injure biomarkers. D) Two hours after de boost dose, the  
556 number of cells in the BAL fluid was counted. Arithmetic means  $\pm$  SD (n = 3). \*\*\* p<0.01  
557 as compared to PBS.

558

559 **Figure 3. Adsorption of LACK-DNA to CCM.** A) Varying amounts of LACK DNA were  
560 incubated with 50 mg of CCM in citrate-phosphate buffer pH 5.5 and ethanol (2:1) for 2  
561 h at 37 °C under agitation. Then, microparticles were centrifuged and the amount of  
562 LACK DNA in the supernatants was determined at 260 nm wavelength. B) Naked CCM  
563 and LACK DNA/CCM (1.2 mg) were stained with ethidium bromide and observed under  
564 fluorescent microscopy. C) Dried microparticles were imaged by SEM in lower (left) and  
565 higher (right) magnification.

566

567 **Figure 4. Uptake of LACK DNA/CCM by the nasal mucosa.** Mice (n = 3) were given  
568 a single i.n. dose of 30  $\mu$ g of LACK DNA/1.2mg CCM in 20  $\mu$ L of PBS (10  $\mu$ L in each  
569 nostril). After 15 min, the nasal mucosa was analyzed in 4  $\mu$ m - histological sections  
570 stained with Masson's trichrome. A) Longitudinal section of the nose of a representative  
571 animal is seen (25 x). B-C) Higher magnification (400 x) of inlets in A. Arrows show  
572 microparticles stained red in the nasal epithelium.

573

574 **Figure 5 - Efficacy of LACK DNA/CCM against cutaneous leishmaniasis.** BALB/c  
 575 mice were given two i.n. instillations with 30 µg of LACK DNA or 30µg LACK DNA/1.2mg  
 576 CCM in 20 µL of PBS (10 µL / nostril) with a 7-day interval. Controls received PBS alone  
 577 or naked CCM in equivalent amounts. Seven days after the booster, animals were  
 578 infected with  $10^5$  *L. amazonensis* promastigotes/footpad, as indicated. A) Lesion sizes  
 579 were measured at the indicated days. B) The parasite loads were evaluated by LDA on  
 580 day 107. Arithmetic means  $\pm$  SD (n=5). \*  $p < 0.05$  and ##  $p < 0.01$  in relation to PBS; \*\*\*  
 581  $p < 0.01$  in relation to both PBS and CCM; ###  $p < 0.01$

582

583 **Figure 6. Peripheral Th1 response expansion by vaccination.** Mice were fully  
 584 vaccinated and infected with *L. amazonensis*, as for Figure 5. A) After 3 days of infection,  
 585 gene expression of transcription factors T-bet, GATA-3 and FoxP3 was evaluated in the  
 586 draining lymph nodes by RT qPCR. GAPDH expression was used as endogenous  
 587 control and results were expressed as relative quantities (RQ) using the PBS-vaccinated  
 588 group for normalization. B) After 107 of infection, IFN- $\gamma$  was measured by ELISA in the  
 589 infected footpad lysates. Results are representative of 3 different experiments. Means  $\pm$   
 590 SEM (n = 5). \*\* $p < 0.05$  (vs. LACK DNA), \*\*\* $p < 0.01$  (vs. non-vaccinated PBS).

591

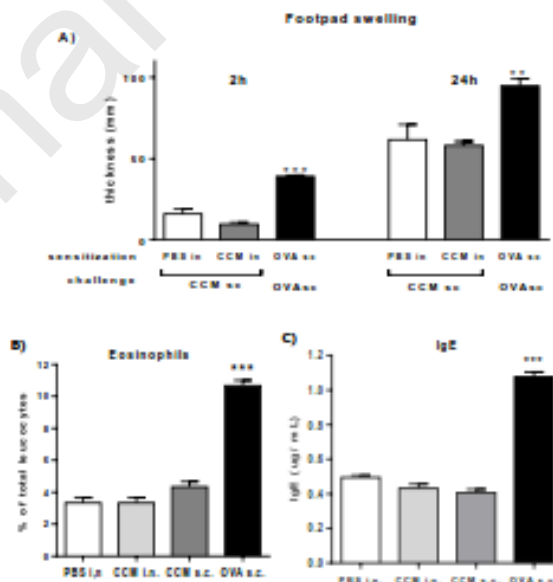
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Figure 1

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Figure 1



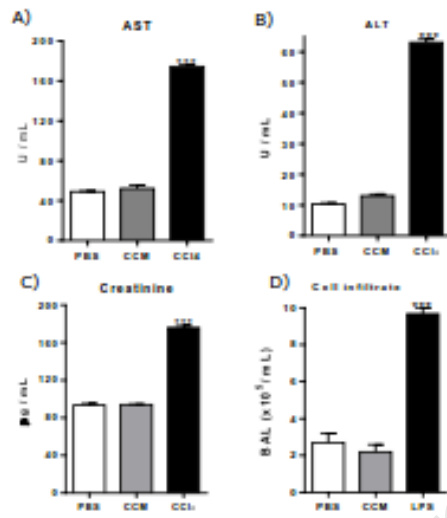
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Figure 2

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Figure 2



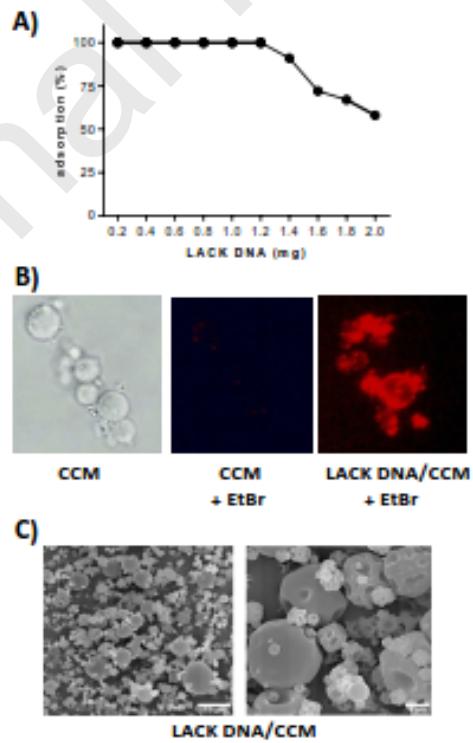
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Figure 3

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Figure 3



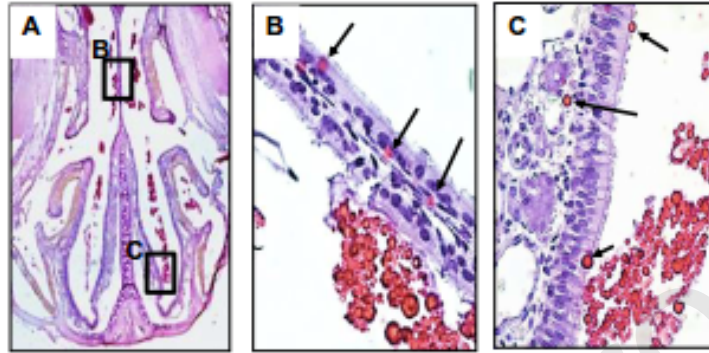
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Figure 4

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Figure 4



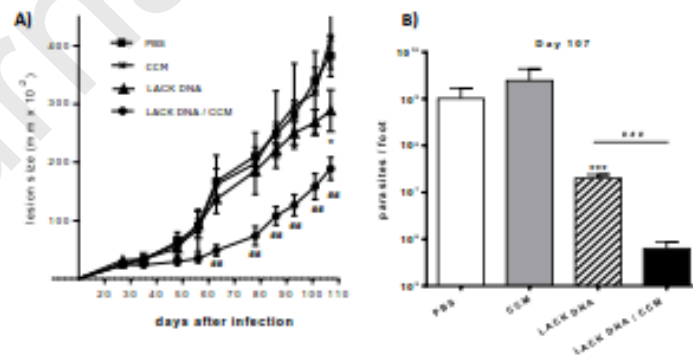
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Figure 5

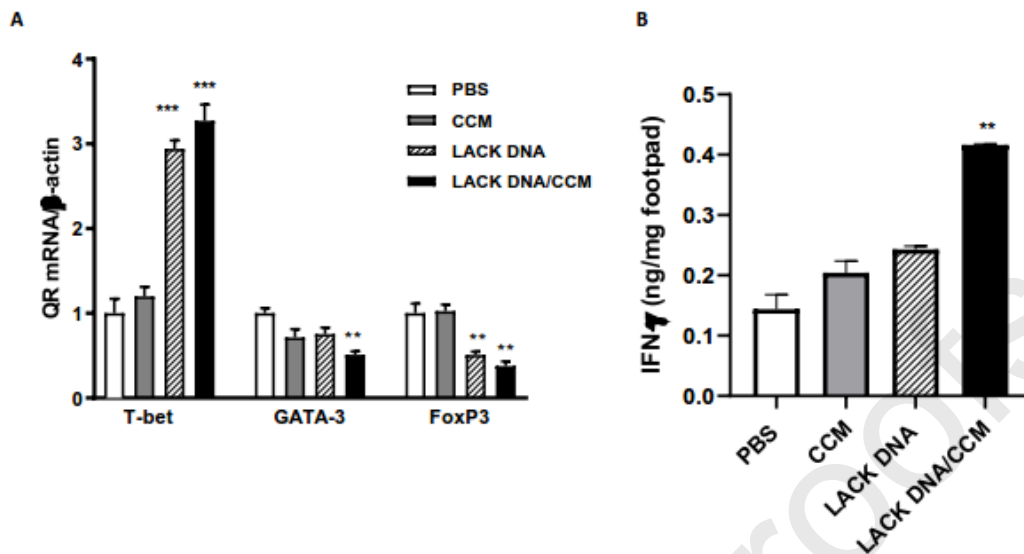
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Figure 5



602

603



604

605 **Abstract**

606

607 Intranasal (i.n.) vaccination with adjuvant-free plasmid DNA encoding the leishmanial  
 608 antigen LACK (LACK DNA) has shown to induce protective immunity against both  
 609 cutaneous and visceral leishmaniasis in rodents. In the present work, we sought to  
 610 evaluate the safety and effectiveness of d,l-glyceraldehyde cross-linked chitosan  
 611 microparticles (CCM) as a LACK DNA non-intumescent mucoadhesive delivery system.  
 612 CCM with 5  $\mu$ m of diameter was prepared and adsorbed with a maximum of 2.4% (w/w)  
 613 of DNA with no volume alteration. Histological analysis of mouse nostrils instilled with  
 614 LACK DNA / CCM showed microparticles to be not only mucoadherent but also  
 615 mucopenetrant, inducing no local inflammation. Systemic safeness was confirmed by the  
 616 observation that two nasal instillations one week apart did not alter the numbers of  
 617 bronchoalveolar cells or blood eosinophils; did not alter ALT, AST and creatinine serum  
 618 levels; and did not induce cutaneous hypersensitivity. When challenged in the footpad  
 619 with *Leishmania amazonensis*, mice developed significantly lower parasite loads as  
 620 compared with animals given naked LACK DNA or CCM alone. **That was accompanied  
 621 by increased stimulation of Th1-biased responses, as seen by the higher T-bet / GATA-  
 622 3 ratio and IFN- $\gamma$  levels.** Together, these results demonstrate that CCM is a safe and  
 623 effective mucopenetrating carrier that can increase the efficacy of i.n. LACK DNA  
 624 vaccination against cutaneous leishmaniasis.

625

626

627 **Declaration of interests**

628

629  The authors declare that they have no known competing financial interests or  
 630 personal relationships that could have appeared to influence the work reported in this  
 631 paper.



632

633 The authors declare the following financial interests/personal relationships which may be  
634 considered as potential competing interests:

635

636

637

638

639

640

641 **Crosslinked chitosan microparticles as a safe and efficient DNA carrier for**  
642 **intranasal vaccination against cutaneous leishmaniasis**

643

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