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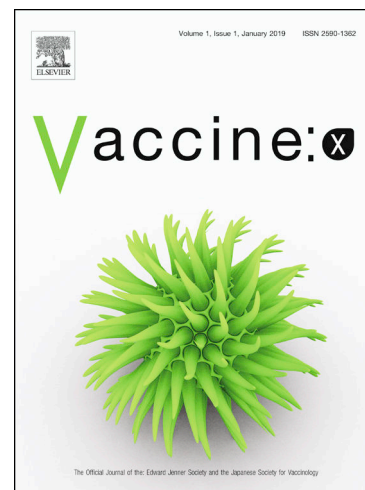
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1 **Crosslinked chitosan microparticles as a safe and efficient DNA carrier for**
2 **intranasal vaccination against cutaneous leishmaniasis**

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 μm 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- γ 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 α TM (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 μ 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 μ L of PBS (10 μ L
145 in each nostril) 7 days apart. Controls received 50 μ 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 μ g of CCM or 50 μ g of
148 OVA in 20 μ 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 μ 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 (CCl_4 , Sigma-Aldrich) in 100 μ 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 μ g of LPS in 20 μ 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 μ g of LACK DNA/1.2 mg
170 CCM in 20 μ 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 μ 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 μ g
178 of naked LACK DNA; 30 μ g LACK DNA/1.2mg CCM (LACK DNA / CCM); or 1.2 mg of
179 naked CCM in 20 μ L of PBS (10 μ 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 μ 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 μ 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- γ was quantitated by ELISA using paired rat IgG1 anti-
213 mouse IFN- γ (clone XMG1.2) and biotinylated rat IgG1 anti-mouse IFN- γ (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 μm 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 μg 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 μ L of PBS (10 μ 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- γ 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

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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 μ L), or LPS (10 μ L) with a 7-day interval, or a single i.p. injection with 1 % CCl₄, 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 μ g of LACK DNA/1.2mg CCM in 20 μ L of PBS (10 μ L in each
 569 nostril). After 15 min, the nasal mucosa was analyzed in 4 μ 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- γ 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

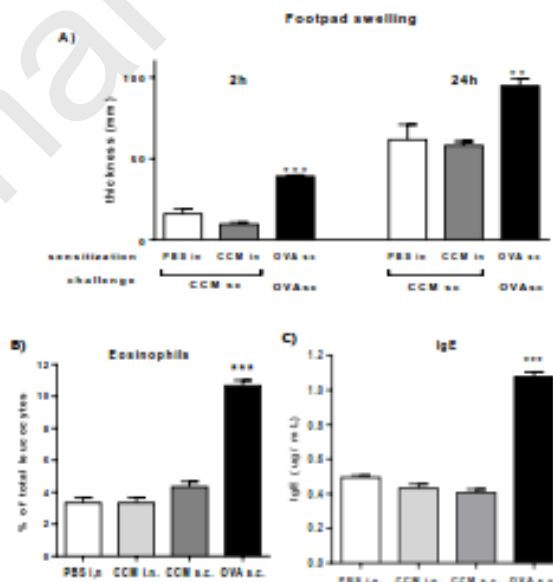
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Figure 1

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Figure 1



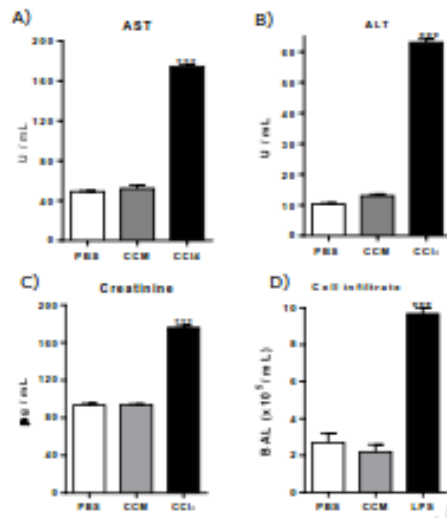
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Figure 2

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Figure 2



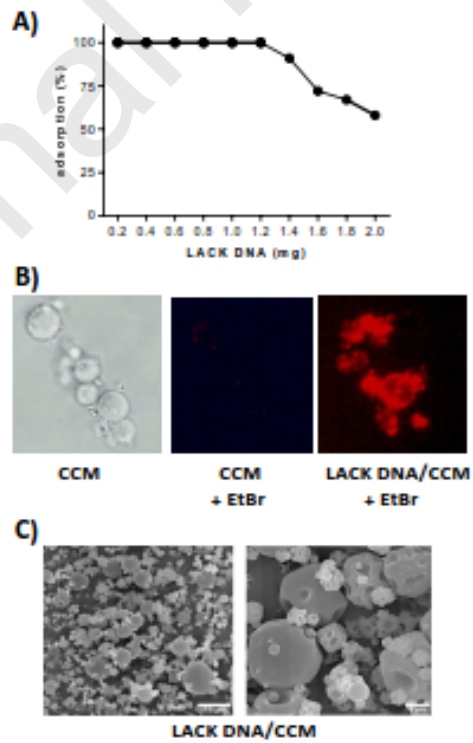
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Figure 3

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Figure 3



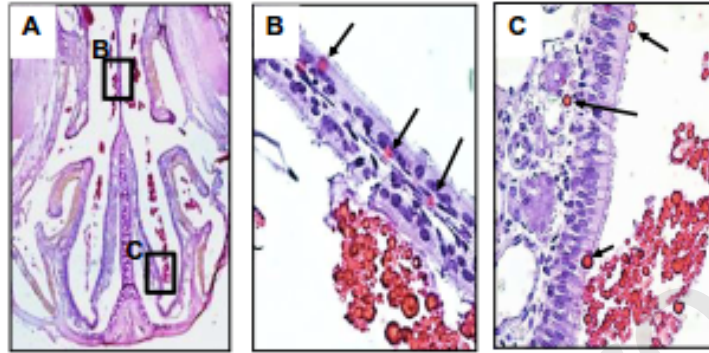
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Figure 4

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Figure 4



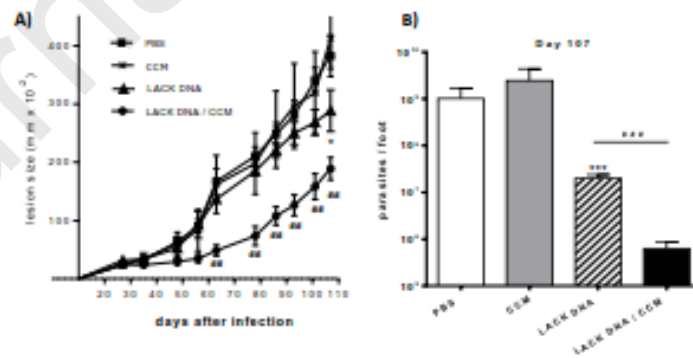
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Figure 5

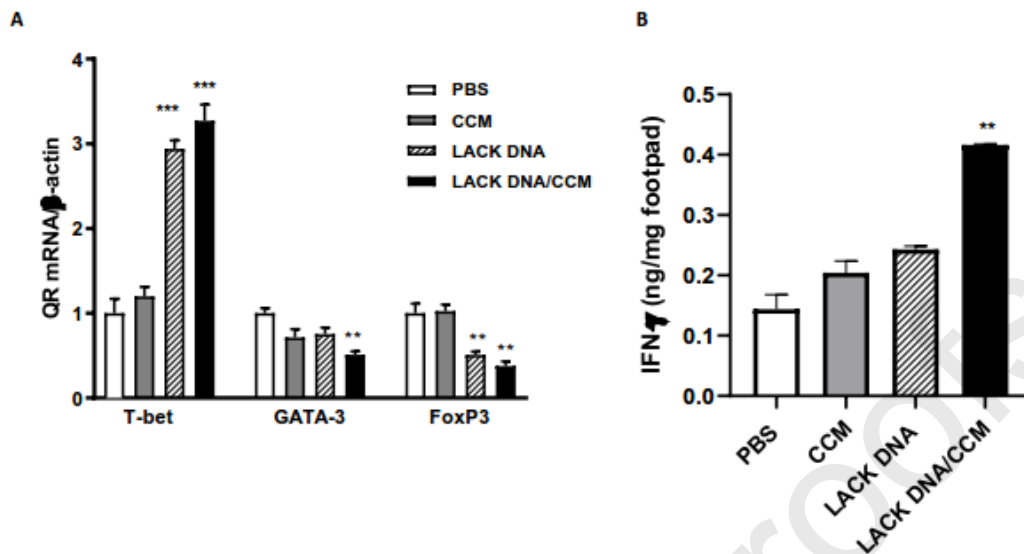
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Figure 5



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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 μ 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- γ 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:

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640

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

643

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