RESEARCH ARTICLE

Oral immunization with heat-inactivated *Mycobacterium bovis* induces protection against infection with *Leishmania amazonensis* in mice

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Abstract

Leishmaniasis is a parasitic infection caused by protozoa of the genus *Leishmania* that affects both humans and animals. It is regarded as one of the major neglected tropical diseases with 1 million new cases occurring annually. It is known that BCG, an attenuated strain of *Mycobacterium bovis* (*M. bovis*), confers partial protection against heterologous pathogens, including *Leishmania*. We investigated if oral immunization with heat-inactivated *M. bovis* (HIMB) would protect mice against *Leishmania* infection. Eighteen female BALB/c mice were randomly assigned to two groups: the immunized group, that received oral HIMB, and the control group. All mice were subsequently infected by inoculation of 10,000 *Leishmania amazonensis* promastigotes in the footpad. At necropsy, spleen culture was positive in all controls and in 55% of mice from the immunized group. In mice belonging to the immunized group, we observed a reduction in the number of immunolabeled amastigotes in the popliteal lymph node ($P = 0.009$) and fewer mature granulomas containing lymphocytes in the liver ($P = 0.005$) as compared to controls. Granulomas from the immunized group presented more Lys+ macrophages ($P = 0.002$) and fewer CD3+ T lymphocytes ($P < 0.001$). Thus, immunization with HIMB via the oral route limited parasite dissemination and granuloma development in mice

45 challenged with *Leishmania amazonensis* through stimulation of the innate immune
46 response involving macrophages. We speculate that, although partial, this heterologous
47 protection matches the concept of trained immunity, which had been mostly attributed to
48 live mycobacteria.

49 *Keywords:* heat-inactivated *Mycobacterium bovis*; *Leishmania*; liver; trained immunity.

50

51 1. Introduction

52 Classically, the innate immune response had been considered unable of mounting immune
53 memory [1]. Nevertheless, growing scientific evidence suggests the occurrence of
54 "innate memory" whereby the innate response against a pathogen is enhanced upon an
55 encounter with the same or another microorganism [2, 3]. Aiming to name this
56 groundbreaking phenomenon, Netea et al., (2011) [4] coined the term "trained
57 immunity". Trained immunity is defined by three main features: a) it is induced after a
58 first contact, through infection or vaccination, with certain microorganisms or their
59 components, b) it confers non-specific cross-protection against a wide range of
60 homologous or heterologous pathogens in an adaptative-independent manner and c) it
61 involves innate cells, receptors, and cytokines, boosting the proinflammatory response.
62 So far, metabolic shifts (from oxidative phosphorylation to aerobic glycolysis) and
63 epigenetic reprogramming (through histone modifications) in monocytes-macrophages
64 [5–7], natural killer (NK) cells [8], dendritic cells [9], and neutrophils [10], as well as in
65 myeloid bone marrow precursors [11], have been recognized as core molecular
66 mechanisms involved in trained immunity. As new insights into the phenomenon of
67 trained immunity are disclosed, vaccines and adjuvants that confer non-specific

68 protection relying on innate responses are increasingly regarded as a promising ally to
69 prevent infectious diseases [11].

70 Leishmaniasis is a parasitic infection caused by protozoa of the genus *Leishmania* that
71 affects both humans and animals [12]. Along with malaria, it is regarded as one of the
72 major neglected tropical diseases [13], with an estimate of up to 1 million new cases
73 occurring annually [12]. Although most infected hosts do not develop clinical disease,
74 *Leishmania* infection may appear as visceral leishmaniasis or kala-azar, which is the most
75 severe form; as cutaneous leishmaniasis, which is the most common form; or as
76 mucocutaneous leishmaniasis [14]. *Leishmania* spp. present a digenetic life cycle, with a
77 flagellated extracellular form (promastigote) in the phlebotomine sandfly vector and an
78 aflagellated intracellular form (amastigote) in the mammalian host, whose target cell is
79 the macrophage [15]. Currently, prevention strategies are based on vector control and host
80 treatment [12], since no vaccine is available for use in humans yet [16]. Conversely, three
81 licensed vaccines against canine leishmaniosis are currently available: Leish-Tec® (Ceva
82 Santé Animale, Libourne, France), CaniLeish (Virbac, Carros cedex, France) and
83 Letifend (Leti Pharma S.L.U.; Madrid, España), based on diverse vaccine development
84 approaches [16]. Vast research aiming to manufacture a licensed vaccine against human
85 leishmaniasis has been conducted, with a few candidates reaching pre-clinical or clinical
86 phase studies [16]. Attempts of vaccination and immunotherapy included whole-killed
87 parasites combined with life attenuated *Mycobacterium bovis* Bacillus Calmette-Guerin
88 (BCG) [17–21].

89 Beyond being the only currently licensed vaccine against tuberculosis (TB), BCG is a
90 well-established inductor of trained immunity [22]. In addition to reducing TB and other
91 mycobacterial diseases symptomatology and pathology, numerous epidemiological

92 studies suggest that the incidence and severity of non-related infections is lower in BCG-
93 vaccinated people (reviewed in [23]). Moreover, experimental evidence supports the
94 hypothesis that BCG confers partial protection against heterologous pathogens, such as
95 certain viruses ([24, 25] but see [26]), bacteria other than mycobacteria [27], fungi [27,
96 28], and intracellular parasites [29, 30] in animal models and in humans. Recently, Silva
97 et al. 2021 [31] deepened in the capacity of BCG to protect against *Leishmania*
98 attributable to trained immunity. Mice vaccinated parenterally with BCG prior to
99 challenge with diverse *Leishmania* species presented lower parasite burden and
100 dissemination, as well as reduced lesion severity.

101 Although not so extensively explored, similar effects have been attributed to
102 immunization with inactivated mycobacteria. Garrido et al. (2011) [32] proposed an
103 immunostimulant containing heat-inactivated *M. bovis* (HIMB) as a complementary tool
104 for TB control in wildlife. Later, Juste et al. (2016) [5] observed a boosted clearance of
105 mycobacteria by macrophages from cattle that had been intramuscularly administered
106 HIMB prior to *in vitro* challenge with *M. bovis*. Further, HIMB has demonstrated not only
107 to confer protection against TB in a wide range of animal species [33–37], but also to
108 elicit partial immunity against non-related bacterial [38], protozoal (Ferreras-Colino et
109 al., submitted), and arthropod (ticks; [39]) infections in different animal models.
110 Specifically, Ferreras-Colino et al. (submitted) observed that immunization with HIMB
111 via the oral route lessened parasite load and liver pathology in mice challenged with
112 another intracellular protozoan, *Plasmodium.*

113 Based on the above, we hypothesized that oral immunization with HIMB would mitigate
114 parasite burden and pathological lesions in *Leishmania* infection through immune
115 mechanisms compatible with trained immunity. Thus, the objective of the present study

was to evaluate the protective effects of HIMB against *Leishmania* infection and to characterize the immunological mechanisms involved.

2. Material and Methods

2.1. Study design

Eighteen female BALB/c mice four weeks old were housed in Instituto de Salud Carlos III (Majadahonda, Madrid) and randomly assigned to two groups (n=9/group): the immunized group, that received orally two doses of HIMB of 50 µl each at an interval of 4 weeks, and the control group, that was administered phosphate-buffered saline (PBS). All mice were subsequently infected by footpad inoculation in the right feet with 10,000 promastigotes of *Leishmania amazonensis* (*L. amazonensis)* in a final volume of 0.05 ml four weeks after the second immunization dose. When the right plantar pad diameter doubled the left one (10 weeks after infection), mice were anesthetized, euthanized, and necropsied (Figure 1). Blood and tissue (footpad, popliteal lymph node (NL), spleen and liver) were obtained for serological, microbiological, histopathological and/or immunohistochemical analysis.

This study was approved by the Ethics Committee of the Instituto de Salud Carlos III (Ref OEBA ISCIII M-02-2015 and CBA ISCIII 10-2015) and the Regional Agriculture Authority (Comunidad de Madrid; permit number: PROEX 114/15). Handling of the animals and sampling were performed according to European (Council Directive 86/609) and Spanish Legislation (RD53/2013).

2.2. Oral HIMB immunostimulant

The oral HIMB consisted of approximately 10^7 heat-inactivated colony forming units (CFU)/ml of a field isolate of *M. bovis* (Strain 1403; SB0339 spoligotype), obtained from

139 a naturally infected wild boar, diluted in sterile PBS. The immunostimulant was prepared
140 following the protocol described by Garrido et al. (2011) [32], except for an extended
141 inactivation step at 83°C for 45 minutes (min). Bacterial concentration of HIMB was
142 determined prior to inactivation by measuring Turbidity in a VITEK® DensiCHEK®
143 (BioMerieux) and by plating a serially diluted aliquot onto agar-solidified Middlebrook
144 7H9 with glycerol (0,2% v/v) and OADC (10% v/v) (Becton Dickinson, Franklin Lakes,
145 NJ, USA).

146 *2.3. Leishmania amazonensis*

147 *Leishmania amazonensis* Maria (MHOM/Br/79/Maria) promastigotes were cultured in
148 RPMI 1640, (Lonza Group, Basel, Switzerland), supplemented with 10% heat-inactivated
149 FCS (Biowest), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin
150 (Lonza Group, Basel, Switzerland). Cultures were incubated at 26°C and submitted to 15
151 passages until they reached the stationary phase. Parasites were harvested by
152 centrifugation (1,200 g, 15 min, 20°C), washed twice in RPMI, and adjusted at $1x10^6$
153 promastigotes/ml.

154 *2.4. Parasite burden by limiting dilution*

155 The *Leishmania* spp. burden by limiting dilution assay as previously described [40].
156 Briefly, spleen and popliteal samples were weighed and homogenized in Schneider's
157 medium (Sigma-Aldrich, Misuri, USA) supplemented with 20% heat-inactivated FBS,
158 2% sterile human urine, 20 mM HEPES, 1% L-glutamine and 100 U/mL penicillin and
159 100 mg/mL streptomycin (Lonza Group, Basel, Switzerland). Organ homogenates were
160 filtered through a cell strainer to ensure a single-cell suspension. Further dilutions of the
161 homogenate were done in the same medium to a final concentration of 10 mg/mL; 200
162 mL/well of this cell suspension was added to the first well of a 96-well culture plate

163 (Corning Inc., NY, USA) and serial 4-fold dilutions were made across the plate. Plates
164 were incubated at 26 ºC for 2 weeks. The presence of parasites was assessed by
165 microscopy after 14 days. The parasite burden (number of parasites/g of organ) was
166 calculated as described in [41] by two blinded and experienced observers (I.M. and M.D.).

167 *2.5. Histopathology*

168 Liver samples were collected during necropsies and fixed in 10% buffered formalin for
169 24 h at RT. Fixed samples were dehydrated in a graded series of ethanol, immersed in
170 xylol, and embedded in paraffin wax using an automatic processor. Sections were cut at
171 3 μm and stained with hematoxylin and eosin (HE) for further histological examinations.

172 The presence and type of mononuclear cell infiltrates and granulomas were evaluated and
173 categorized following the criteria described in [42]. Mononuclear cell infiltration was
174 labeled as parenchymal/interstitial, if located in the liver parenchyma, or periportal, if
175 located in the periportal space. Granulomas were classified according to the number of
176 cells in small (<25 cells) or big (>25 cells) granulomas and according to shape and cellular
177 components in immature (irregular non-rounded containing hepatocytes and erythrocytes
178 in the center and more Kupffer cells and macrophages than lymphocytes, and with or
179 without amastigotes), mature (regular rounded containing more lymphocytes than
180 macrophages and without hepatocytes and erythrocytes) and clean granulomas (rounded
181 with collagen capsule or spiral containing more lymphocytes than macrophages).
182 Infiltrates were graded as absent or 0% (0), mild or 10% to 30% of liver parenchyma
183 affected (1), moderate or 30% to 70% affected (2), and severe or 70% to 100% affected
184 (3); while granulomas were counted in 20 randomly chosen 0.2 mm^2 fields per slide.
185 Pathology was independently evaluated by two blinded and experienced observers (E.F-
186 C. and M.A.R.).

187 *2.6. Immunohistochemistry*

188 Immunohistochemistry was performed using the avidin-biotin-peroxidase complex
189 (ABC) method to detect *Leishmania* amastigotes in the inoculated footpad, popliteal NL,
190 spleen and liver fixed in 10% buffered formalin and embedded in paraffin wax.
191 Furthermore, immune cells (neutrophils, macrophages, T lymphocytes, regulatory T
192 lymphocytes, B lymphocytes) and immune mediators (iNOS, IFN, TNF, IL-1, IL-6) in
193 the liver were assessed with the same technique. Briefly, tissue sections (3 μm) were
194 dewaxed and rehydrated through a graded ethanol series. Then, endogenous peroxidase
195 activity was exhausted by incubation with 0.3% hydrogen peroxide in methanol for 30
196 min at RT. After 3 rinses of 10 min each in PBS (pH 7.2), samples were subjected to
197 different pretreatments for antigen retrieval depending on the primary antibodies (Abs)
198 used (Table 1). Primary Abs produced in mouse were used with the M.O.M.® (Mouse on
199 Mouse) Immunodetection Kit (Vector Laboratories, Burlingame, USA) following
200 manufacturer's instructions. Subsequently, slides were rinsed 3 times in PBS for 10 min
201 and covered with 1% normal rabbit serum (Pierce-Endogen, Woburn, MA) for the rat
202 primary Abs or 20% normal goat serum (Thermo Fisher Scientific, Whaltman, MA) for
203 the rabbit primary Abs in PBS for 30 min at RT. Then, tissue sections were incubated
204 overnight at 4ºC with the primary Abs. The following day, slides were washed in PBS (3
205 times for 5 min each) and incubated with the secondary Abs for 30 min at RT.
206 Biotinylated rabbit anti–rat IgG secondary Ab (Dako, Glostrup, Denmark) diluted 1:200
207 in PBS containing normal rabbit serum 1% was used for the rat primary Abs, biotinylated
208 goat anti–rabbit IgG secondary Ab (Vector Laboratories, Burlingame, CA) diluted 1:200
209 in PBS containing normal goat serum 10% was used for the rabbit primary Abs and
210 M.O.M. Biotinylated Anti-Mouse IgG Reagent for the mouse Ab (Vector Laboratories,
211 Burlingame, USA). After 3 washes of 5 min each in PBS, slides were incubated with the

212 ABC complex (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, USA) for 1
213 hour (h) at RT. Then, all tissue sections were rinsed in 0.05 M Tris buffered saline (TBS;
214 pH 7.6) and incubated with the chromogen solution (NovaRED Substrate Kit; Vector
215 Laboratories, Burlingame, USA) until color reveal. Finally, slides were counterstained
216 with Harris's hematoxylin.

217 *Leishmania* PCR-positive experimentally infected mice's footpad was used as positive
218 control for the *Leishmania* immunohistochemistry, whereas disease-free mice lymphoid
219 tissue was used as positive control for the immune cells and cytokines
220 immunohistochemistry. For negative control, specific primary Abs were replaced by Ab
221 isotype control or rabbit non-immune sera (DakoCytomation, Glostrup, Denmark).

222 All granulomas in the slide were examined to quantify the number of cells per granuloma
223 to 0.2 mm^2 fields. Cells were identified based on shape, size and positive immunolabeling
224 and counted by two experienced observers (E.F.-C. and M.A.R.).

225 *2.7. IgG isotypes against Leishmania spp.*

226 Sera were obtained from blood samples by centrifugation (3000 *g* for 10 min) and the
227 peripheral specific antileishmanial Ab response was determined by indirect ELISA.
228 Briefly, microtitre 96-well plates (Thermo Fisher Scientific, Whaltman, MA) were coated
229 with 50 µg/mL of soluble *L. amazonensis* antigen overnight at 4ºC.The wells were
230 subsequently washed with a PBS solution containing 0.05% Tween-20 (PBST) and
231 blocked with 2% BSA at 37ºC for 1 h (75 µl/well). Animal sera were used at dilution
232 1/100 (50 µL/well), as established by prior ELISA chequerboard titrations, and incubated
233 for 2 h at 37ºC. After 3 washes, secondary Abs were added and the plates were incubated
234 for 30 min at room temperature (RT). Abs were diluted in PBS at 1/2000 dilution for total
235 IgG, IgG1, IgG2a, IgG2b, IgG3 and IgM) [Goat Anti-mouse immunoglobulin HRP,

236 Southern Biotech]. Wells were washed three times with PBST and the enzyme reaction
237 initiated with 100 µL/well HRP substrate (1 mg/mL OPD in citrate-phosphate buffer
238 pH 4.8, with 33% H_2O_2 diluted 1/1000). The reaction was terminated by adding
239 50 µL/well 3 N H_2SO_4, and the product measured by absorbance (A 492nm) in a
240 microplate reader (Anthos 2020, Salzburg, Austria).

241 *2.8. Statistical analysis*

242 Data were assessed to calculate mean ± standard deviation (SD) or error (SE) values and
243 were analyzed with the SPSS statistical software package (V.24.0; IBM, Somers, New
244 York, USA). A prior Shapiro-Wilk test was performed to assess the normality of data. As
245 data were not normally distributed, differences in parasite burden, pathological findings,
246 immune mediators and cells, and IgG isotypes between the immunized group and the
247 control group were tested through a Mann-Whitney U test. P values ≤ 0.05 were
248 considered statistically significant.

249 3. Results

250 *Oral immunization with HIMB reduces organic dissemination of L. amazonensis*

251 The thickness of the footpad inoculated with *L. amazonensis* was monitored weekly until
252 it doubled the thickness of the non-inoculated footpad (at day 60 post-infection). Parasite
253 burden was assessed at the end of the experiment by popliteal LN and spleen culture, as
254 well as footpad, spleen, popliteal LN, and liver immunohistochemistry. Infection was
255 confirmed by detection of promastigotes in spleen and popliteal LN culture and of
256 immunolabeled amastigotes in the inoculated footpad.

257 Promastigotes appeared at day 7 of culture in both groups. After 14 days of culture,
258 promastigotes were detected in popliteal LN culture from all animals, while spleen culture
259 was positive in 100% of animals from the control group (mean: 132.64 promastigotes)
260 and 55.55% of animals from the immunized group (mean: 87.62 promastigotes)
261 (P=0.082). No between-group differences were observed in the popliteal LN culture and
262 the spleen promastigote burden (P>0.05).

263 Amastigotes were observed inside macrophages that were diffusely distributed in the
264 footpad which had been inoculated with *L. amazonensis* and inside macrophages that
265 were multifocally distributed in the associated popliteal LN (Figure 2B and C). Although
266 no differences were observed between groups regarding the footpad, a significant
267 reduction in the number of immunolabeled amastigotes in the popliteal LN of the
268 immunized group compared with the control group was detected (P = 0.009) (Figure 2A).
269 Of note, no amastigotes were observed in the liver or spleen of any of the mice.

270 *Oral immunization with HIMB slows granuloma development in L. amazonensis*
271 *infection*

272 Major pathological findings observed in animals from both groups were mononuclear cell
273 infiltrates and granulomas. Infiltrates were mainly composed of lymphocytes and
274 macrophages and were present both at the portal space and in the parenchyma. The extent
275 of the parenchymal infiltrates was significantly larger in the immunized group compared
276 with the control group (P = 0.006), whereas no differences in periportal infiltrates were
277 observed (Figure 3 and 4). Granulomas consisted in rounded lesions in the parenchyma
278 containing macrophages and lymphocytes. The number of mature granulomas containing
279 more lymphocytes was lower in the immunized group compared with the control group
280 (P = 0.005), while no differences between groups were observed regarding granuloma

size (Figure 3 and 4). Of note, no amastigotes were observed in the infiltrates nor in the granulomas of either group.

HIMB stimulates macrophages response against L. amazonensis

Granulomas from the immunized group presented more Lys+ macrophages (P = 0.002) and fewer CD3+ T lymphocytes (P < 0.001) compared with the control group, along with fewer neutrophils. CD79+ B lymphocytes were scarce in all granulomas and no differences between groups were observed (Figure 5). A scarce expression of cytokines was detected in the liver granulomas from both groups, except for a high, although non-significant, iNOS production in immunized animals (Figure 6).

HIMB does not influence the humoral response against L. amazonensis

The humoral response was modest and with predominance of immunoglobulin G, especially isotype IgG1 (data not shown). No significant differences between groups in immunoglobulin levels nor in immunoglobulin isotypes were observed.

4. Discussion

Overall, we demonstrated that HIMB administered via the oral route confers cross-protection against *L. amazonensis* in mice. Altogether, the decrease in parasite load in popliteal LNs as well as the less severe liver pathology in immunized animals indicate that HIMB hampers parasite dissemination and slows down lesion onset and growth during *Leishmania* infection. To the best of our knowledge, comparable effects against leishmaniasis attributed to an immunostimulant composed of inactivated mycobacteria had not been reported in the literature yet.

302 However, certain weaknesses of our study ought to be mentioned. Foremost, animal
303 experimentation principles imply reducing the number of animal replicates and the
304 duration of the infection period, thereby limiting the statistical power and the assessment
305 of long-term effects. Also, since immune responses against *Leishmania* infection vary
306 among parasite and host species, our findings might not be entirely extrapolated to other
307 models [43], such as human leishmaniasis due to *L. donovani* and canine leishmaniasis
308 due to *L. infantum.* However, we observed clear differences between the immunized and
309 the control groups which suggest that our findings with the mouse-*L. amazonensis* model
310 might be indicative of the possible effects on other host-*Leishmania* binomia.

311 Despite numerous attempts, developing an effective and safe vaccine against protozoal
312 infections in humans remains a major challenge in vaccine research [44]. Although
313 several vaccines against *L. infantum* in dogs are currently available, the limited protection
314 reported under field conditions (reviewed in [45, 46]) encourages the seek for novel
315 antigen and adjuvant candidates. For instance, a study comparing a vaccine candidate
316 (multicomponent LBSap) with two commercial vaccines against canine leishmaniasis
317 (Leish-Tec® and Leishmune®) reported a comparable reduction of *Leishmania* burden
318 in the spleen and the liver of mice vaccinated and challenged with *L. infantum*, regardless
319 of the vaccine, although only the Leish-Tec® group displayed a monocyte response after
320 infection [47]. Furthermore, another vaccine candidate (recombinant LiUBC1 protein)
321 achieved a total clearance of *L. infantum* in the spleen of immunized hamsters [48].

322 The beneficial effect of BCG against *Leishmania* infection has been long known [13].
323 Epidemiological retrospective studies in humans have suggested a reduced incidence of
324 leishmaniasis in children vaccinated with BGC [49], whereas experimental studies in
325 rodent models have demonstrated a reduction in parasite burden in target organs of

326 animals that had been vaccinated with BCG prior to challenge with *Leishmania* [31, 50,
327 51]. Moreover, Silva et al. (2021) [31] demonstrated that intravenous administration of
328 BCG also reduces lesion size in mice infected with several *Leishmania* species, in
329 association with increased inflammatory infiltrate in infected tissues and with improved
330 parasite killing through increased production of reactive oxygen species by BCG-trained
331 monocytes. In the present study, an increased capacity of the host innate immunity to
332 control *Leishmania* infection due to stimulation with HIMB was observed. This is
333 illustrated by the macrophage response and iNOS production rather than a specific
334 response against the parasite led by lymphocytes [31, 47]. Of note, in the referred works,
335 the vaccine was administered via the parenteral route. Therefore, our study opens the door
336 for an innovative approach by using the oral route and an inactivated mycobacterium.

337 Despite the successful experience of immunotherapy and chemotherapy with BCG in
338 humans [19–21, 52], live mycobacteria cannot be considered a safe adjuvant in the case
339 of leishmaniasis vaccination, since individuals may develop a local abscess at the
340 inoculation site several weeks after subcutaneous administration [51, 53]. Considering
341 the abovementioned data, as well as the present work, we support the use of inactivated
342 mycobacteria, such as HIMB, as novel adjuvant candidates to combine with both
343 commercial and under-research vaccines. Further studies using both *in vitro* and *in vivo*
344 approaches would be of great interest to deepen in the extent of host protection against
345 the parasite, as well as in the molecular mechanisms involved in each animal species. For
346 instance, stimulating human monocyte-derived macrophages with HIMB prior to
347 challenge with *L. donovani* would constitute a step in its potential application for
348 developing a vaccine against human leishmaniasis; whereas testing the protection against
349 *L. infantum* in dogs vaccinated with a licensed vaccine combined with HIMB would allow
350 upgrading the efficacy and safety of available vaccines against canine leishmaniasis.

351 In conclusion, immunization with HIMB via the oral route slows down local parasite
352 dissemination and hepatic granuloma development in mice infected with *L. amazonensis*
353 through stimulation of the innate response involving macrophages. Although partial, the
354 heterologous protection described in the present work matches the concept of “trained
355 immunity”, which had been mostly attributed to live mycobacteria. Thus, HIMB ought to
356 be further explored as an alternative adjuvant in vaccine development.

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538

539 **Figure captions**

540 **Figure 1.** Experimental design. BALB/c mice were randomly assigned to two groups of
541 9 animals each. The immunized group received two doses (50 μl each) of HIMB via oral
542 route at an interval of 4 weeks, and the control group received phosphate-buffered saline
543 (PBS) instead. All animals were inoculated 10.000 *L. amazonensis* promastigotes at the
544 right footpad one month after the second immunization dose. Ten weeks after inoculation,
545 when the right footpad diameter doubled the left one, mice were anesthetized, euthanized,
546 and necropsied.

547 **Figure 2.** Parasite burden. Mean ± SE of *Leishmania* burden detected by
548 immunohistochemistry in the popliteal lymph node (A). Representative fields of the
549 immunized (B) and control (C) group at end of the experiment. Statistically significant
550 differences between groups: (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.00$; Mann-Whitney U test for
551 non-parametric distributions).

552 **Figure 3**. Histopathological findings associated to *Leishmania* infection in the liver.
553 Mononuclear cell infiltrates in the hepatic parenchyma (blue arrowheads and blue arrow)
554 and granulomas (black arrowhead and black arrow) in the hepatic parenchyma of
555 immunized (A and C) and control (B and D) mice at the end of the experiment. The
556 immunized group presented more mononuclear cell infiltrate (blue arrowhead) and more
557 immature granulomas (black arrowhead), whereas the control group presented lesser
558 mononuclear cell infiltrate (blue arrow) and more mature granulomas (black arrow).
559 Hematoxylin-eosin stain.

560 **Figure 4.** Histopathological lesions associated to *Leishmania* infection. Mean ± SE of
561 parenchymal (A) and periportal (B) infiltrate score in the liver of the immunized and the
562 control groups at end of the experiment. Mean ± SD of percentage of immature and
563 mature (C), small and big (D) granulomas in the liver of the immunized and the control

564 groups at end of the experiment. Statistically significant differences between both groups:

565 (*P≤0.05, **P≤0.01, ***P≤0.00; Mann-Whitney U test for non-parametric distributions).

566 **Figure 5.** Mean ± SE of Gr1+ neutrophils (A) lysozyme+ macrophages (B), CD3+

567 lymphocytes T (C), CD79+ lymphocytes B (D) number in granulomas in the liver of the

568 immunized and the control groups at end of the experiment. Statistically significant

569 differences between both groups: (*P≤0.05, **P≤0.01, ***P≤0.00; Mann-Whitney U test

570 for non-parametric distributions).

571 **Figure 6.** Mean ± SE of iNOS+ macrophages number in granulomas in the liver of both

572 groups (A) at end of the experiment. The number of iNOS+ macrophages in granulomas

573 was higher in the immunized group (B) compared with the control group (C).

574

575 **Tables.**

576 **Table 1.** Details of the immunohistochemical primary antibodies.

Specificity	Antigen or cell detected	Species of origin	mAb/pAb	Dilution	Source
Leishmania spp.	Leishmania spp. amastigotes	Rabbit	pAb	1/50	IS Carlos III
Gr1[a]	Neutrophiles	Rat	mAb (clone RB6-8C5)	1:100	Biolegend®
Lysozyme[a]	Macrophages	Rat	pAb	1:200	Dako/Agilent
CD3[b]	T lymphocytes	Rabbit	pAb	1:100	Biolegend®
CD79 [c]*	B Lymphocytes	Mouse	pAb	1:25	Dako/Agilent
iNOS[c]	iNOS	Rabbit	pAb	1:100	BD Biosciencies
IFN-γ[d]*	IFN-γ	Mouse	mAb (clone CC302)	1:10	Bio-Rad AbD Serotec
TNF-α[e]	TNF-α	Rabbit	pAb	1:25	Bio-Rad AbD Serotec
IL-1α[f]	IL-1	Rabbit	pAb	1:100	Endogen
IL-6[e]*	IL-6	Mouse	mAb (clone 4B6)	1:50	Bio-Rad AbD Serotec

577 mAb: monoclonal antibody; pAb: polyclonal antibody.

578 [a]Incubation with 0.2% proteinase K (Sigma-Aldrich, Misuri, USA) in Tris buffer at 37ºC
579 for 8 min.

580 [b]Incubation with 0.1 M tri-sodium citrate dihydrate (TC; Merck KGaA, Darmstadt,
581 Alemania) pH 6, microwave at sub-boiling temperature for 6 min.

582 [c]Incubation with 0.1 M TC pH 6, autoclave at 121ºC for 6 min.

583 [d]Incubation with 0.1 M TC pH 3.2; microwave at sub-boiling temperature for 6 min.

584 [e]Incubation with 0.1 M TC pH 3.2 at 37ºC for 30 min.

585