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1 **FULL-LENGTH PAPER**

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3 # Impact of primary mouse macrophage cell types on *Leishmania*
4 # infection and *in vitro* drug susceptibility.

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9

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26 **ABSTRACT**

27 Primary mouse macrophages are frequently used to provide an *in vitro* intracellular model to
28 evaluate antileishmanial drug efficacy. The present study compared the phenotypic characteristics of
29 Swiss, BALB/c and C57BL/6 mouse bone marrow-derived macrophages and peritoneal exudate cells
30 using different stimulation and adherence protocols upon infection with a *Leishmania infantum*
31 laboratory strain and two clinical isolates. Evaluation parameters were susceptibility to infection,
32 permissiveness to amastigote multiplication and impact on drug efficacy. Observed variations in
33 infection of peritoneal exudate cells can mostly be linked to changes in the inflammatory cytokine
34 profiles (IL-6, TNF-α, KC/GRO) rather than to differences in initial production of nitric oxide and
35 reactive oxygen species. Optimization of the cell stimulation and adherence conditions resulted in
36 comparable infection indices amongst peritoneal exudate cells and the various types of bone
37 marrow-derived macrophages. Noteworthy is that BALB/c-derived bone marrow-derived
38 macrophages were slightly more permissive to intracellular amastigote replication. Evaluation of
39 antileishmanial drug potency in the various cell systems revealed minimal variation for antimonials
40 and paromomycin, but no differences for miltefosine and amphotericin B. The study results allows to
41 conclude that drug evaluation can be performed in all tested primary macrophages as only marginal
42 differences were observed in terms of susceptibility to infection and impact of drug exposure.
43 Combined with some practical considerations, the use of 24h starch-stimulated, 48h-adhered, Swiss-
44 derived peritoneal exudate cells can be advocated as an efficient, reliable, relatively quick and cost-
45 effective tool for routine drug susceptibility testing *in vitro* whenever the use of primary cells is
46 feasible.

47

48 **Introduction**

49 Primary mouse macrophages are considered to be a very robust and biologically relevant model to
50 evaluate drug efficacy against *Leishmania*. Unfortunately standardization of protocols is still lacking,
51 resulting in a wide variety of cell manipulation protocols and cell types [(1)]. Linked to the susceptible
52 genetic profile of BALB/c mice to *Leishmania* infection, BALB/c-derived macrophages are often used
53 in *in vitro* assays. In various infection models where C57BL/6 mice exhibit higher resistance than
54 BALB/c mice, these differences have been ascribed to differential macrophage responses *in vitro* and
55 *in vivo* [(2-5)]. For cutaneous leishmaniasis (CL), the comparison of macrophages from various mouse
56 strains and the associated *in vitro* immunological responses have been evaluated in relation to the
57 clear Th1/Th2 dichotomy determining control of CL *in vivo* [(6, 7)]. Similar studies are scarce for visceral
58 leishmaniasis (VL) as this dichotomy is not observed. However, it was shown that macrophages
59 isolated from different tissues possess a diverse basal metabolic programming and can therefore
60 react differently to pathogens [(8)]. Next to using different mouse strains, both bone marrow-derived
61 macrophages (BMMϕ) and peritoneal exudate cells (PECs) are currently used for *Leishmania*
62 susceptibility assays. PECs are generally isolated after intraperitoneal stimulation with either 2%
63 starch or 3% sodium thioglycolate. Although the collection of macrophages from the peritoneal
64 cavity can be carried out without prior stimulation, it is generally included to increase the amount of
65 collected cells. However, while the stimulation itself could cause a differential immunological
66 macrophage activation, the stimulation period may also impact on the subtypes of immune cells that
67 are collected, which could alter the susceptibility to *Leishmania* infection and drug action [(9, 10)]. Given
68 the prevailing need for assay harmonization [(1)], this study made an in-depth phenotypic and
69 immunological comparison of PECs and BMMϕ from Swiss, BALB/c and C57BL/6 mice exposed to *L.*
70 *infantum* parasites. In the search of the optimal *in vitro* model to determine antileishmanial drug
71 susceptibility of clinical isolates, four aspects were evaluated: (i) origin of the macrophages, (ii)
72 mouse strain, (iii) stimulation time prior to PEC collection and (iv) cell adherence time.

73

74 **Material and methods**

75 Ethics statement: The use of laboratory rodents was carried out in strict accordance to all mandatory
76 guidelines (EU directives, including the Revised Directive 2010/63/EU on the Protection of Animals
77 used for Scientific Purposes that came into force on 01/01/2013, and the declaration of Helsinki in its
78 latest version) and was approved by the ethical committee of the University of Antwerp, Belgium
79 (UA-ECD 2016–54 (02/09/2016)).

80 Laboratory animals: Female Swiss, BALB/c and C57BL/6 mice (body weight 15-20g) for the collection
81 of BMMϕ and PECs were purchased from Janvier (Le Genest Saint Isle, France). All animals were kept
82 on a regular rodent diet (Carfil, Arendonk, Belgium) and drinking water was available *ad libitum*.

83 Parasites and *in vitro* cultures: As it was the aim to develop an optimal *in vitro* model to determine
84 antileishmanial drug susceptibility of clinical isolates, all infections were carried out using metacyclic
85 *Leishmania* promastigotes. The phenotypic and immunological experiments were carried out with
86 the reference laboratory *L. infantum* strain (MHOM/MA/67/ITMAP263). The phenotypic findings
87 were further validated on the recent *L. infantum* clinical isolates MHOM/FR/95/LEM3049 and
88 MCAN/BR/2002/BH402/60. The latter is a canine isolate from Brazil that was part of a large efficacy
89 trial with a liposomal meglumine-antimoniate formulation [(11)]. The drug-susceptible LEM3049 isolate
90 was obtained from a HIV-positive patient from the Languedoc area in France, and was also used to
91 evaluate the more subtle host-dependent variations in drug susceptibility given its higher overall
92 susceptibility to most drugs (*Supplementary data*). Parasites were routinely cultured in T25 culture
93 flasks containing 5 mL of HOMEM (Invitrogen, UK) supplemented with 10% heat inactivated fetal
94 bovine serum (iFBS). One day prior to infection, promastigotes were preconditioned in HOMEM at pH
95 5.4, as described previously [(12)].

96 Collection of peritoneal macrophages: Primary peritoneal exudate cells (PECs) were collected from
97 female Swiss, BALB/c and C57BL/6 mice, as described previously [(13)]. Since most studies with PECs use
98 either starch or thioglycolate stimulation to attract macrophages to the abdominal cavity and
99 increase overall yield, different stimulation periods were compared. Mice were stimulated with a 2%

100 starch suspension in PBS either 48h or 24h prior to macrophage collection. To evaluate the
101 phenotypic and immunological effects of the stimulation process, macrophages derived from non-
102 stimulated mice were included as controls. Thioglycolate stimulation was not considered here as
103 previous studies already demonstrated significant alterations of physiological macrophage properties
104 [(14)]. After collection, macrophages were counted and plated in 96-well plates at a concentration of
105 30,000 cells/well for the phenotypic evaluation. For NO measurements, cells were seeded at 800,000
106 cells/well while for the detection of ROS a concentration of 10^6 cells per well was adopted in a 24
107 well plate. The different types of PECs were incubated at 37°C in presence of 5% CO_2 and were
108 allowed to adhere for 1h, 24h or 48h before infection with metacyclic promastigotes.

109 Collection of bone marrow-derived macrophages: Bone marrow-derived macrophages (BMMϕs)
110 were obtained from the femur and tibia of female Swiss, BALB/c and C57BL/6 mice by flushing the
111 bone cavities with cold RPMI-1640 medium. The bone marrow was kept on ice during the isolation,
112 then centrifuged for 20 minutes upon which the red blood cells were lysed with an ammonium-
113 chloride-potassium lysis buffer (VWR, Leuven, Belgium). The recovered cells were resuspended in
114 RPMI-1640 medium supplemented with 1% non-essential amino acids, 1% penicillin-streptomycin
115 solution, 1% sodium pyruvate, 1% L-glutamine, 10% iFBS and 15% L929 supernatant containing
116 macrophage colony stimulating factor (M-CSF), and incubated in Petri dishes at 37°C and 5% CO_2 for 7
117 days in order to obtain macrophage monolayers. The cells were detached from the dishes with
118 dissociation buffer (1% 0.5M EDTA, 2% 1M HEPES in PBS) 24h before infection, counted in KOVA®
119 counting slides and seeded in 96-well plates in RPMI-1640 medium supplemented with 5% bovine
120 serum, 2% penicillin-streptomycin and 1% L-glutamine at final concentrations similar to those used
121 for the PECs.

122 Receptivity for infection and intracellular amastigote multiplication: Macrophages were infected with
123 metacyclic promastigotes at a ratio of 10:1. For each condition, the intracellular parasite burden was
124 quantified microscopically in at least of 50 macrophages, from which the infection index (average
125 number of amastigotes/cell) was calculated [(15)]. Infection indices at 24h post-infection (hpi) were

126 used to compare susceptibility to infection of the different macrophage host cells. The permissive
127 capacity for infection was evaluated by assessing intracellular parasite replication over time, as
128 described previously [(16)]. In brief, intracellular amastigote burdens were assessed microscopically
129 every 24h up to 168 hpi following methanol fixation and Giemsa-staining . As the initial receptivity
130 for infection generally aligned well with the degree of intracellular amastigote replication, the 24hpi
131 infection index was used to define the optimal conditions for the *in vitro* model.

132 Evaluation of drug susceptibility: Macrophages were infected with preconditioned promastigotes of
133 the LEM3049 clinical isolate at a ratio of 10:1 and exposed 24h later for 96h to two-fold drug
134 dilutions of pentavalent (Sb^V) and trivalent (Sb^{III}) antimonials, miltefosine (MIL), paromomycin (PMM)
135 and amphotericin B (AmB) (Fungizone®). After methanol fixation and Giemsa-staining, the 50%
136 inhibitory concentration (IC_{50}) was determined microscopically by comparing the infection ratio
137 between the treated and the untreated infected control cells.

138 Measurement of ROS production: Electron paramagnetic resonance (EPR) experiments were carried
139 out to evaluate the O_2^- production in the collected macrophages with and without *Leishmania*
140 infection. After a 24h attachment phase, the culture medium was removed and replaced by Krebs-
141 Henseleit buffer (KHB) mixed with 25 µM of deferoxamine and 5 µM sodium diethyldithiocarbamate
142 trihydrate (DETC) at pH 7.4. The cell permeable ROS sensitive spin probe CMH (1-hydroxy-3-
143 methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine) was added at 1 mM and incubated for 50 minutes
144 at room temperature. The supernatant was then immediately loaded into a 50 µL glass capillary
145 (Duran Ringcaps, Hirschmann, Germany) for measurement in the table-top MiniScope MS 200
146 spectrometer (Magenttech Germany). Five scans of 30 sec each were accumulated for each sample
147 at a center field of 3350 G at room temperature. Modulation amplitude was set at 2000 G with an
148 attenuation of 15dB and a gain of 10. Results were saved and evaluated using the Analysis 2.0
149 software (Magnettech, Germany) by calculating the amplitude as a measure of free radical
150 concentration in the sample. Results are expressed as a percentage ratio compared to PECs from
151 unstimulated Swiss mice. To evaluate ROS production after infection, cells were infected with

152 preconditioned metacyclic promastigotes at an infection ratio of 10:1 parasites per macrophage in
153 KHB mixed buffer. After 5 minutes of incubation, CMH was added at a final concentration of 1mM for
154 50 minutes. EPR spectra were acquired as described above.
155 Measurement of NO production: A Griess reagent kit (Promega G2930) was used for the
156 determination of extracellular nitrite in infected macrophages. To avoid interference, RPMI-1640
157 without phenol red was used. After 24h of attachment, the cells were infected with preconditioned
158 metacyclic promastigotes at an infection ratio of 10:1. After 48h of incubation, the supernatant was
159 transferred to a 96-well plate (150µL/well) with the addition of 20 µL Griess reagent and 130 µL
160 demineralized water. Samples were incubated for 30 min in the dark and absorbance was measured
161 at 550nm (Labsystems Multiskan MCC/340). A standard calibration curve was set up by diluting the
162 nitrite standard of the kit.
163 Cytokine production: To assess whether immunological differences between the various cell types
164 may underline the observed phenotypic variations, the production of a cytokine subset with a known
165 involvement in active leishmaniasis was evaluated by multiplex ELISA. Supernatants of the different
166 cell types were collected 48h after infection and kept at -80°C until analysis. Cytokines were
167 quantified in a V-plex custom mouse cytokine plate (IL-1β, IL-6, KC/GRO, TNF-α and IL-10) using the
168 multiplex electro-chemiluminescent detection Quickplex SQ120 system (Meso Scale Discovery)
169 following to the manufacturer's instructions.
170 Statistical analysis: All statistical analyses were performed using Graphpad Prism version 4.00
171 software. Statistical differences between the different macrophage types and between the different
172 conditions within one group were determined using 2-way ANOVA with Bonferroni post-hoc
173 comparisons. Intergroup comparisons were done using non-parametric Friedman test followed by
174 Dunn's post-hoc comparisons. Tests were considered statistically significant if $p < 0.05$.
175

176 **Results**

177 Impact of starch stimulation and PEC adherence time on *L. infantum* infection

178 The susceptibility of PECs from the various mouse genetic backgrounds (Swiss, BALB/c and C57BL/6)
179 to *L. infantum* infection and the impact of starch stimulation and cell adherence time was based on
180 the mean number of intracellular amastigotes at 24hpi for the different PEC types. For all tested
181 parasite strains, the highest infection indices were reached in the starch stimulated PECs, most
182 clearly in PECs derived from Swiss and C57BL/6, while BALB/c PECs showed an intrinsically higher
183 infection susceptibility without a significant benefit from the stimulation (Figure 1).
184 The impact of cell adhesion time prior to infection (1h, 24h or 48h) on macrophage infection was also
185 evaluated at 24hpi. For all tested strains, infection indices increased with the adherence time of
186 Swiss and C57BL/6 PECs (Figure 2). As for the starch stimulation, prolonged adherence of the
187 intrinsically more susceptible BALB/c PECs did not enhance but rather decreased parasite infection
188 burdens. In summary, the highest infection indices were reached using the optimal combination of
189 24h starch stimulation and 48h cell adherence for both Swiss- and C57BL/6-derived PECs. The highest
190 infection burdens in BALB/c-derived PECs were achieved with 24h starch stimulation and only 1h cell
191 adhesion. PECs collected according to these optimized conditions and bone marrow-derived
192 macrophages were subsequently compared for overall susceptibility to infection and amastigote
193 multiplication.

194

195 Impact of cell source on infection and intracellular amastigote multiplication

196 PECs isolated under the optimal conditions of starch stimulation and cell adherence for each strain
197 did not reveal significant differences in the infection indices (Figure 3A). When comparing the
198 different BMMϕ with the PECs, no statistical differences could be detected. C57BL/6 BMMϕ seemed
199 slightly less susceptible to infection (Figure 3B).

200

201 Although marginal differences could be observed between the PECs from the various mouse strains,
202 the overall intracellular amastigote replication profiles were fully comparable (Figure 4A+B+C). The
203 clinical LEM3049 strain was clearly showing intracellular amplification in PECs as compared to the
204 limited or no expansion of the ITMAP263 and BH402/60 strains. Swiss and C57BL/6- BMMϕ harbored
205 fully comparable parasite burdens. Remarkably, BALB/c-derived BMMϕ supported heavier
206 amastigote replication, even for the strains that did not expand significantly in PECs (Figure 4D+E+F).
207

208 Immunological observations correlate with the observed differences in infectivity
209 ROS, NO and cytokine production were assessed in relation to the observed differences in cell
210 infectivity and intracellular amastigote proliferation. The increased susceptibility to infection upon
211 prolonged adherence of C57BL/6 PECs correlated with significantly lower ROS production (Figure 5),
212 whereas ROS levels in PECs of BALB/c and Swiss mice remained unaltered. No statistical differences
213 could be detected in ROS production of BMMϕ originating from the various mouse strains. No
214 biologically relevant changes in NO production were observed in both PECs and BMMϕ of Swiss,
215 BALB/c and C57BL/6 mice.
216 The pronounced increase in susceptibility of starch stimulated Swiss PECs could be linked to overall
217 lower basal levels of the tested cytokines and a less prominent TNF-α and KC/GRO response to
218 infection (Figure 6). In C57BL/6 PECs, the increased parasite burdens upon prolonged adherence
219 correlated with low infection-triggered TNF-α and KC/GRO production (Figure 7, Figures S1 - S2).
220 Prolonged adherence of BALB/c-derived PECs provoked elevated basal and infection-induced
221 cytokine levels corresponding with the observed lower infection indices.
222 Despite the clear difference in amastigote multiplication in BALB/c as compared to Swiss and
223 C57BL/6 BMMϕ, no differential cytokine response profile could be associated with this phenomenon
224 (Figure 8).
225
226

227 Impact of cell source on antiparasitic drug susceptibility profiling

228 The drug susceptibility (IC_{50}-values) of intracellular amastigotes for the panel of antileishmanial
229 reference drugs (Sb^V, Sb^{III}, MIL, PMM and AmB) was examined for each cell type (Table 1). Small
230 shifts in susceptibility depending on the different starch stimulation periods and cell adherence times
231 can be observed. Although drug susceptibility for Sb^{III}, MIL and AmB did not significantly vary
232 between the various PECs, some differences could be observed for Sb^V and PMM. The Sb^V
233 susceptibility in Swiss- and BALB/c-derived PECs decreased as a result of starch induction, which
234 actually coincides with higher intrinsic parasite burdens. Similarly, the higher infection indices in
235 BALB/c PECs following shorter cell adherence times coincided with decreased drug susceptibility.
236 Some variation was observed for PMM which mostly related to the starch stimulation. In general, *L.*
237 *infantum* amastigotes were found to be more resistant to Sb^V and Sb^{III} in BMMϕ than in PECs. The
238 higher intracellular amastigote burdens in BALB/c BMMϕ did not result in major changes in the drug
239 efficacy results, but correlated to the relatively lower IC_{50} value for PMM as compared to the one
240 obtained in PECs ($p<0.05$).

241

242

243 **Discussion**

244 Various host cell types have been used for *in vitro* drug susceptibility evaluation of *Leishmania.*
245 Although some laboratories may have no access to primary cells and remain restricted to the use of
246 commercially available cell lines, it is generally well-accepted that primary macrophages provide a
247 more optimal and robust intracellular model that has advantages in terms of biological relevance.
248 Research comparing primary peritoneal mouse macrophages (PECs), mouse bone marrow-derived
249 macrophages (BMMϕ) and human blood monocyte-derived macrophages (PBMCs) already indicated
250 differences in drug susceptibility dependent on the host cell type [10]. Despite these findings, different
251 cell types are still being widely used for drug screening and susceptibility evaluation, highlighting a
252 need for assay harmonization. Furthermore, the collection of primary macrophages can be
253 performed adopting diverse procedures, including various mouse strains and following different
254 stimulation and cell adherence protocols. The present study on primary macrophages specifically
255 focused on comparing the phenotypic and immunological consequences of these variables on
256 promastigote-induced infection, support of intracellular parasite expansion and drug susceptibility.
257 Firstly, the production of superoxide (O_2^-) and nitric oxide (NO) upon parasite infection was evaluated
258 as these are known key factors in the establishment of infection. The initial increase in O_2^- occurring
259 when promastigotes are phagocytosed by macrophages [17] has been shown to be toxic for
260 promastigotes [18, 19], while NO as a second oxidant is mostly effective in killing the intracellular
261 parasite generally after macrophage activation by IFN-γ and TNFα [20-22]. Although the parasite has
262 developed several defense strategies to overcome the detrimental effects of O_2^- and NO, such as
263 intracellular thiols and $T(SH)_2$ [23, 24], some of the current antileishmanial reference drugs have been
264 shown to counteract these defense mechanisms and increase the intracellular NO and/or O_2^-
265 production [25-27]. Recognizing the pivotal role of NO and O_2^- , using an intracellular *in vitro* model
266 where both are produced *in situ* appears imperative for appropriate drug susceptibility assays. Cell
267 adherence was also found to be an important parameter determining the susceptibility of PECs to *L.*
268 *infantum* infection. NO and O_2^- measurements only revealed a decreased ROS production in C57BL/6

269 PECs upon prolonged adherence and was linked to an increased susceptibility to infection. Starch
270 stimulation induced higher infections in Swiss PECs, but could not be linked to biologically relevant
271 differences in ROS and NO production. The observation of a markedly increased intracellular parasite
272 survival in BALB/c BMMϕ corroborated earlier studies linking host immunology to the cell's
273 phenotype *in vitro* for other pathogens [2, 3, 28], but was not reflected by a lower NO or O_2^- production
274 in the present study.

275 Secondly, several cytokines have already been implicated in driving the infection dynamics of
276 *Leishmania in vivo* [29, 30]. In animal models, the disease outcome is shown to be determined by a
277 delicate innate balance between several pro- and anti-inflammatory cytokines [31]. Numerous studies
278 investigated the immunological responses of macrophages to *L. major* infection, with only limited
279 induction of gene expression [32]. Earlier research reported the induction of several pro-inflammatory
280 cytokines such as IL-1β, TNF-α and IL-6 upon phagocytosis of *Leishmania* parasites[33-35], while the
281 production of anti-inflammatory cytokines such as IL-10 has been described as well [36]. Although IL-
282 10 is a known suppressor of inflammatory cytokine production, thereby exacerbating disease
283 progression, its production upon parasite infection in this *in vitro* study was minimal and is in
284 agreement with previous research [34, 37]. Past research also linked the production of IL-10 to a
285 decreased NO production and reduced parasite killing [38], but no correlation with the observed
286 differences in macrophage infection could be demonstrated with the VL strains used in the present
287 study. The increasing infection rates upon starch-stimulation of the Swiss PECs and upon longer cell
288 adherence of Swiss- and C57BL/6-derived PECs was found to be linked to a decreased pro-
289 inflammatory KC/GRO and TNF expression profile, known to regulate the control of *Leishmania*
290 infection [33-35]. KC/GRO is the CXCL1 chemokine that contributes *in vivo* to the recruitment of
291 macrophages and neutrophils to the site of infection [32] and TNF is a known inducer of leishmanicidal
292 activity in the macrophage. Beside a lower KC/GRO and TNF responsiveness, the beneficial effect of
293 starch stimulation on parasite receptivity of Swiss PECs was also linked to a decreased basal
294 activation status, *e.g.* overall lower levels of IL-10, IL-6, KC/GRO and TNF-α. It is assumed that the

295 observed differences in cytokine profile are associated with various other phenomena such as
296 membrane trafficking, the parasite's access to nutrients, apoptosis or autophagy that all may affect
297 *Leishmania* growth [(39-41)]. In addition to increasing the infection ratio upon infection, starch
298 stimulation also resulted in a higher cell yield during peritoneal macrophage collection hereby
299 significantly diminishing the number of laboratory animals needed.
300 The findings obtained with the reference laboratory strain ITMAP263 were validated for the recent
301 *L. infantum* clinical isolates LEM3049 and BH402/60. Overall, the main conclusions were comparable
302 for all strains. Slight inter-strain differences, however, indicate that starch stimulation and cell
303 adherence conditions could require some degree of individual fine-tuning when aiming to produce
304 maximal infection burdens. Next to the observed inter-strain differences, an impact of the infecting
305 parasite-stage on infectivity and intracellular infection dynamics can be anticipated as well. While
306 this study used metacyclic promastigotes for infection, conditions of starch stimulation and cell
307 adherence might require some modifications when working with *ex vivo*-derived amastigotes.
308 Although the phenotypic and immunological implications of the adopted protocols are important
309 criteria to select the optimal conditions for *in vitro* drug susceptibility evaluation, ensuing practical
310 issues should be considered as well. Collection of BMMϕ is complex, requires more steps and is more
311 time-consuming because bone marrow-progenitor cells should be differentiated into macrophages
312 by addition of M-CSF to the culture medium [(42)]. BALB/c BMMϕ appeared to be the most susceptible
313 cell type for supporting intracellular amastigote amplification of all parasite strains tested. In contrast
314 to the tedious process of obtaining BMMϕ, the collection of high numbers of macrophages from the
315 peritoneal cavity is far more straightforward and will reduce the number of required laboratory
316 animals, a highly relevant factor when responsibly conducting animal experiments. Although the
317 activation state of the macrophages may influence drug susceptibility, the *in vitro* drug susceptibility
318 values obtained in the various cell systems were quite comparable (Table 1). The recorded decrease
319 in susceptibility to Sb^V with increasing parasite burdens is not unexpected given that similar
320 observations were made in a previous report [(43)]. Using the outbred Swiss mouse for the collection of

cells also provides a far cheaper alternative than using special inbred mouse strains, such as BALB/c or C57BL/6.

To conclude, the evaluation of drugs in the different primary macrophages can generally be performed on all cell types as only marginal differences are observed in terms of susceptibility to infection. These phenotypic observations can mostly be linked to differences in the cytokine profile rather than to differences in initial NO and ROS production upon parasite infection. These nominal phenotypic differences combined with some practical considerations favor the use of 24h starch-stimulated, 48h-adhered, Swiss-derived PECs as an efficient, relatively quick and cheap tool for routine drug susceptibility testing *in vitro* whenever the use of primary cells is feasible.

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340 **References**

341 1. **Hendrickx S, Guerin PJ, Caljon G, Croft SL, Maes L.** 2016. Evaluating drug resistance in visceral
342 leishmaniasis: the challenges. Parasitology**:**1-11.
343 2. **Kaushik RS, Uzonna JE, Zhang Y, Gordon JR, Tabel H.** 2000. Innate resistance to experimental
344 African trypanosomiasis: differences in cytokine (TNF-alpha, IL-6, IL-10 and IL-12) production by
345 bone marrow-derived macrophages from resistant and susceptible mice. Cytokine **12:**1024-
346 1034.
347 3. **Santos JL, Andrade AA, Dias AA, Bonjardim CA, Reis LF, Teixeira SM, Horta MF.** 2006.
348 Differential sensitivity of C57BL/6 (M-1) and BALB/c (M-2) macrophages to the stimuli of IFN-
349 gamma/LPS for the production of NO: correlation with iNOS mRNA and protein expression. J
350 Interferon Cytokine Res **26:**682-688.
351 4. **Howes A, Taubert C, Blankley S, Spink N, Wu X, Graham CM, Zhao J, Saraiva M, Ricciardi-**
352 **Castagnoli P, Bancroft GJ, O'Garra A.** 2016. Differential Production of Type I IFN Determines the
353 Reciprocal Levels of IL-10 and Proinflammatory Cytokines Produced by C57BL/6 and BALB/c
354 Macrophages. J Immunol **197:**2838-2853.
355 5. **Depke M, Breitbach K, Dinh Hoang Dang K, Brinkmann L, Salazar MG, Dhople VM, Bast A, Steil**
356 **L, Schmidt F, Steinmetz I, Volker U.** 2014. Bone marrow-derived macrophages from BALB/c and
357 C57BL/6 mice fundamentally differ in their respiratory chain complex proteins, lysosomal
358 enzymes and components of antioxidant stress systems. J Proteomics **103:**72-86.
359 6. **Velasquez LG, Galuppo MK, E DER, Brandao WN, Peron JP, Uliana SR, Duarte MI, Stolf BS.**
360 2016. Distinct courses of infection with *Leishmania (L.) amazonensis* are observed in BALB/c,
361 BALB/c nude and C57BL/6 mice. Parasitology **143:**692-703.
362 7. **Schmid M, Zimara N, Wege AK, Ritter U.** 2014. Myeloid-derived suppressor cell functionality
363 and interaction with *Leishmania major* parasites differ in C57BL/6 and BALB/c mice. Eur J
364 Immunol **44:**3295-3306.
365 8. **Artyomov MN, Sergushichev A, Schilling JD.** 2016. Integrating immunometabolism and
366 macrophage diversity. Semin Immunol **28:**417-424.
367 9. **Ghosn EE, Cassado AA, Govoni GR, Fukuhara T, Yang Y, Monack DM, Bortoluci KR, Almeida SR,**
368 **Herzenberg LA, Herzenberg LA.** 2010. Two physically, functionally, and developmentally distinct
369 peritoneal macrophage subsets. Proc Natl Acad Sci U S A **107:**2568-2573.
370 10. **Seifert K, Escobar P, Croft SL.** 2010. *In vitro* activity of anti-leishmanial drugs against *Leishmania*
371 *donovani* is host cell dependent. JAntimicrobChemother **65:**508-511.
372 11. **da Costa Val A.** 2004. Tratamento da leishmaniose visceral canina com antimonial pentavalente
373 encapsulado em lipossomas. Tese de Doutorado em Ciência Animal, Escola de Veterinária,
374 Universidade Federal de Minas Gerais, Belo Horizonte, 125p.
375 12. **Inocencio da Luz RA, Vermeersch M, Dujardin JC, Cos P, Maes L.** 2009. *In vitro* sensitivity testing
376 of *Leishmania* clinical field isolates: preconditioning of promastigotes enhances infectivity for
377 macrophage host cells. Antimicrob Agents Chemother **53:**5197-5203.
378 13. **Vermeersch M, da Luz RI, Tote K, Timmermans JP, Cos P, Maes L.** 2009. *In vitro* susceptibilities
379 of *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference drugs:
380 practical relevance of stage-specific differences. Antimicrob Agents Chemother **53:**3855-3859.
381 14. **Hoover DL, Nacy CA.** 1984. Macrophage activation to kill *Leishmania tropica*: defective
382 intracellular killing of amastigotes by macrophages elicited with sterile inflammatory agents. J
383 Immunol **132:**1487-1493.

15. **Hendrickx S, Leemans A, Mondelaers A, Rijal S, Khanal B, Dujardin JC, Delputte P, Cos P, Maes L.** 2015. Comparative Fitness of a Parent *Leishmania donovani* Clinical Isolate and Its Experimentally Derived Paromomycin-Resistant Strain. PLoS One **10:**e0140139.
16. **Hendrickx S, Mondelaers A, Eberhardt E, Lachaud L, Delputte P, Cos P, Maes L.** 2015. Intracellular amastigote replication may not be required for successful *in vitro* selection of miltefosine resistance in *Leishmania infantum*. ParasitolRes **114:**2561-2565.
17. **Gantt KR, Goldman TL, McCormick ML, Miller MA, Jeronimo SM, Nascimento ET, Britigan BE, Wilson ME.** 2001. Oxidative responses of human and murine macrophages during phagocytosis of *Leishmania chagasi*. J Immunol **167:**893-901.
18. **Zarley JH, Britigan BE, Wilson ME.** 1991. Hydrogen peroxide-mediated toxicity for *Leishmania donovani chagasi* promastigotes. Role of hydroxyl radical and protection by heat shock. J Clin Invest **88:**1511-1521.
19. **Miller MA, McGowan SE, Gantt KR, Champion M, Novick SL, Andersen KA, Bacchi CJ, Yarlett N, Britigan BE, Wilson ME.** 2000. Inducible resistance to oxidant stress in the protozoan *Leishmania chagasi*. J Biol Chem **275:**33883-33889.
20. **Evans TG, Thai L, Granger DL, Hibbs JB, Jr.** 1993. Effect of *in vivo* inhibition of nitric oxide production in murine leishmaniasis. J Immunol **151:**907-915.
21. **Diefenbach A, Schindler H, Donhauser N, Lorenz E, Laskay T, MacMicking J, Rollinghoff M, Gresser I, Bogdan C.** 1998. Type 1 interferon (IFNalpha/beta) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. Immunity **8:**77-87.
22. **Cunha FQ, Assreuy J, Xu D, Charles I, Liew FY, Moncada S.** 1993. Repeated induction of nitric oxide synthase and leishmanicidal activity in murine macrophages. Eur J Immunol **23:**1385-1388.
23. **Ghosh S, Goswami S, Adhya S.** 2003. Role of superoxide dismutase in survival of *Leishmania* within the macrophage. Biochem J **369:**447-452.
24. **Barr SD, Gedamu L.** 2003. Role of peroxidoxins in *Leishmania chagasi* survival. Evidence of an enzymatic defense against nitrosative stress. J Biol Chem **278:**10816-10823.
25. **Sarkar A, Saha P, Mandal G, Mukhopadhyay D, Roy S, Singh SK, Das S, Goswami RP, Saha B, Kumar D, Das P, Chatterjee M.** 2011. Monitoring of intracellular nitric oxide in leishmaniasis: its applicability in patients with visceral leishmaniasis. Cytometry A **79:**35-45.
26. **Mookerjee Basu J, Mookerjee A, Sen P, Bhaumik S, Sen P, Banerjee S, Naskar K, Choudhuri SK, Saha B, Raha S, Roy S.** 2006. Sodium antimony gluconate induces generation of reactive oxygen species and nitric oxide via phosphoinositide 3-kinase and mitogen-activated protein kinase activation in *Leishmania donovani*-infected macrophages. Antimicrob Agents Chemother **50:**1788-1797.
27. **Mandal G, Wyllie S, Singh N, Sundar S, Fairlamb AH, Chatterjee M.** 2007. Increased levels of thiols protect antimony unresponsive *Leishmania donovani* field isolates against reactive oxygen species generated by trivalent antimony. Parasitology **134:**1679-1687.
28. **Tabel H, Kaushik RS, Uzonna JE.** 2000. Susceptibility and resistance to *Trypanosoma congolense* infections. Microbes Infect **2:**1619-1629.
29. **Maspi N, Abdoli A, Ghaffarifar F.** 2016. Pro- and anti-inflammatory cytokines in cutaneous leishmaniasis: a review. Pathog Glob Health **110:**247-260.
30. **Rodrigues V, Cordeiro-da-Silva A, Laforge M, Silvestre R, Estaquier J.** 2016. Regulation of immunity during visceral *Leishmania* infection. Parasit Vectors **9:**118.
31. **Loeuillet C, Banuls AL, Hide M.** 2016. Study of *Leishmania* pathogenesis in mice: experimental considerations. Parasit Vectors **9:**144.

429 32. **Racoosin EL, Beverley SM.** 1997. *Leishmania major*: promastigotes induce expression of a
430 subset of chemokine genes in murine macrophages. Exp Parasitol **85:**283-295.
431 33. **Arena A, Capozza AB, Delfino D, Iannello D.** 1997. Production of TNF alpha and interleukin 6 by
432 differentiated U937 cells infected with *Leishmania major.* New Microbiol **20:**233-240.
433 34. **Lapara NJ, 3rd, Kelly BL.** 2010. Suppression of LPS-induced inflammatory responses in
434 macrophages infected with *Leishmania*. J Inflamm (Lond) **7:**8.
435 35. **Lima-Junior DS, Costa DL, Carregaro V, Cunha LD, Silva AL, Mineo TW, Gutierrez FR, Bellio M,**
436 **Bortoluci KR, Flavell RA, Bozza MT, Silva JS, Zamboni DS.** 2013. Inflammasome-derived IL-1beta
437 production induces nitric oxide-mediated resistance to *Leishmania*. Nat Med **19:**909-915.
438 36. **Teixeira MJ, Teixeira CR, Andrade BB, Barral-Netto M, Barral A.** 2006. Chemokines in host-
439 parasite interactions in leishmaniasis. Trends Parasitol **22:**32-40.
440 37. **Denys A, Udalova IA, Smith C, Williams LM, Ciesielski CJ, Campbell J, Andrews C, Kwaitkowski**
441 **D, Foxwell BM.** 2002. Evidence for a dual mechanism for IL-10 suppression of TNF-alpha
442 production that does not involve inhibition of p38 mitogen-activated protein kinase or NF-kappa
443 B in primary human macrophages. J Immunol **168:**4837-4845.
444 38. **Gazzinelli RT, Oswald IP, James SL, Sher A.** 1992. IL-10 inhibits parasite killing and nitrogen
445 oxide production by IFN-gamma-activated macrophages. J Immunol **148:**1792-1796.
446 39. **Matte C.** 2016. Exploitation of the Host Cell Membrane Fusion Machinery by Leishmania Is Part
447 of the Infection Process. **12**.
448 40. **Crauwels P, Bohn R, Thomas M, Gottwalt S, Jäckel F, Krämer S, Bank E, Tenzer S, Walther P,**
449 **Bastian M, Zandbergen Gv.** 2015. Apoptotic-like *Leishmania* exploit the host´s autophagy
450 machinery to reduce T-cell-mediated parasite elimination. Autophagy **11:**285-297.
451 41. **Lima Maciel BL, Valverde JG, Rodrigues-Neto JF, Freire-Neto F, Keesen TSL, Jeronimo SMB.**
452 2014. Dual Immune Modulatory Effect of Vitamin A in Human Visceral Leishmaniasis. PLOS ONE
453 **9:**e107564.
454 42. **Weischenfeldt J, Porse B.** 2008. Bone Marrow-Derived Macrophages (BMM): Isolation and
455 Applications. CSH Protoc **2008:**pdb.prot5080.
456 43. **Fernández O, Diaz-Toro Y, Valderrama L, Ovalle C, Valderrama M, Castillo H, Perez M, Saravia**
457 **NG.** 2012. Novel Approach to *In Vitro* Drug Susceptibility Assessment of Clinical Strains of
458 *Leishmania spp*. Journal of Clinical Microbiology **50:**2207-2211.
459

460 **Table**

Mice	Cell conditioning	Sb^V (eq.)	Sb^{III} (eq.)	Miltefosine (µM)	Paromomycin (µM)	Fungizone® (µM)
	Primary peritoneal mϕ					
Swiss mice	**48h adherence**					
	48h stimulation	30.7 ± 7.8	6.0 ± 1.9	0.7 ± 0.1	64.8 ± 22.3	0.06 ± 0.01
	24h stimulation	**52.4 ± 24.6**	**6.3 ± 0.4**	**0.5 ± 0.0**	**99.3 ± 10.3**	**0.05 ± 0.01**
	no stimulation	38.1 ± 30.5	8.1 ± 4.5	0.8 ± 0.2	63.2 ± 4.2	0.02 ± 0.01
	24h adherence					
	48h stimulation	44.6 ± 32.4	7.5 ± 1.3	0.5 ± 0.0	50.5 ± 5.0	0.05 ± 0.01
	24h stimulation	52.1 ± 24.9	3.9 ± 0.6	0.6 ± 0.0	56.6 ± 13.0	0.04 ± 0.01
	no stimulation	10.3 ± 2.4	5.8 ± 1.7	0.5 ± 0.1	33.1 ± 3.1	0.02 ± 0.01
	1h adherence					
	48h stimulation	74.7 ± 2.4	5.6 ± 1.4	0.5 ± 0.0	82.8 ± 24.7	0.03 ± 0.01
	24h stimulation	44.3 ± 18.9	7.4 ± 2.5	0.6 ± 0.1	62.7 ± 11.8	0.02 ± 0.00
	no stimulation	25.7 ± 8.6	5.2 ± 1.6	0.6 ± 0.1	41.3 ± 7.0	0.04 ± 0.01
BALB/c mice	**48h adherence**					
	48h stimulation	30.3 ± 24.2	2.8 ± 0.8	0.5 ± 0.0	31.8 ± 4.8	0.04 ± 0.02
	24h stimulation	40.4 ± 28.2	6.1 ± 1.8	0.5 ± 0.0	83.3 ± 26.9	0.04 ± 0.02
	no stimulation	15.7 ± 9.7	6.7 ± 3.3	0.6 ± 0.1	85.1 ± 19.9	0.02 ± 0.00
	24h adherence					
	48h stimulation	65.7 ± 11.3	3.9 ± 0.7	0.5 ± 0.0	40.5 ± 5.5	0.04 ± 0.02
	24h stimulation	42.5 ± 11.9	3.5 ± 0.6	0.4 ± 0.1	47.2 ± 8.1	0.06 ± 0.02
	no stimulation	24.8 ± 0.6	2.7 ± 0.4	0.5 ± 0.0	72.2 ± 7.8	0.03 ± 0.00
	1h adherence					
	48h stimulation	77.0 ± 0.0	8.3 ± 3.0	0.6 ± 0.1	66.0 ± 7.5	0.04 ± 0.00
	24h stimulation	77.0 ± 0.0	5.3 ± 1.3	0.5 ± 0.0	73.3 ± 20.0	0.07 ± 0.02
	no stimulation	72.8 ± 4.2	5.3 ± 1.7	0.5 ± 0.0	67.9 ± 11.1	0.04 ± 0.00
C57BL/6 mice	**48h adherence**					
	48h stimulation	31.5 ± 14.3	2.5 ± 0.5	0.7 ± 0.2	44.3 ± 4.3	0.02 ± 0.01
	24h stimulation	46.4 ± 30.6	4.7 ± 1.3	0.7 ± 0.1	57.0 ± 10.0	0.04 ± 0.00
	no stimulation	16.2 ± 14.4	7.9 ± 4.2	0.6 ± 0.1	59.2 ± 23.1	0.06 ± 0.03
	24h adherence					
	48h stimulation	68.4 ± 8.6	8.3 ± 2.7	0.8 ± 0.2	172.2 ± 69.9	0.03 ± 0.01
	24h stimulation	22.4 ± 8.8	8.5 ± 3.1	0.7 ± 0.1	96.6 ± 24.0	0.04 ± 0.00
	no stimulation	44.1 ± 12.5	2.0 ± 0.1	0.6 ± 0.1	107.1 ± 24.7	0.06 ± 0.00
	1h adherence					
	48h stimulation	77.0 ± 0.0	6.8 ± 1.7	0.8 ± 0.2	54.0 ± 1.4	0.06 ± 0.01
	24h stimulation	52.0 ± 16.7	6.2 ± 1.5	0.7 ± 0.2	99.7 ± 35.4	0.06 ± 0.02
	no stimulation	43.5 ± 7.3	4.9 ± 1.5	0.5 ± 0.0	45.0 ± 13.2	0.04 ± 0.01
	Bone marrow-derived mϕ					
	Swiss	77.0 ± 0.0	8.8 ± 0.5	0.9 ± 0.0	88.3 ± 34.1	0.07 ± 0.02
	BALB/c	77.0 ± 0.0	11.1 ± 1.5	0.5 ± 0.1	25.3 ± 7.0	0.05 ± 0.02
	C57BL/6	77.0 ± 0.0	10.9 ± 1.8	0.4 ± 0.1	60.6 ± 5.9	0.07 ± 0.03

461 **Table 1: Drug susceptibility values (IC_{50}) against antileishmanial reference drugs for each cell type.**
462 Cells were infected with the laboratory L. infantum strain ITMAP263. Results are expressed
463 as the mean IC_{50} ± standard error of mean (SEM) and are based on two independent
464 replicates run in duplicate. Results (shaded) correspond to the starch stimulation and
465 adherence conditions resulting in the highest parasite burdens.

466 **Figures**

467

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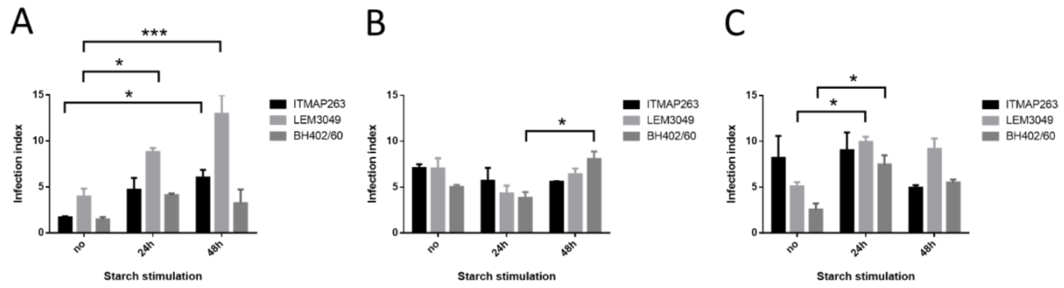

469

470 **Figure 1: Effect of starch stimulation on the infection index in peritoneal exudate cells (PECs) from Swiss (A), BALB/c (B)**
471 **and C57BL/6 mice (C).** Mice were either left unstimulated (no) or were stimulated with 2% starch 24h or 48h
472 prior to collection of PECs. The cells were allowed to adhere 24h before infection. The infection indices were
473 determined 24h post infection. Results are expressed as mean ± standard error of mean (SEM) and are based on
474 two independent replicate runs in duplicate (* $p<0.05$; *** $p<0.001$).

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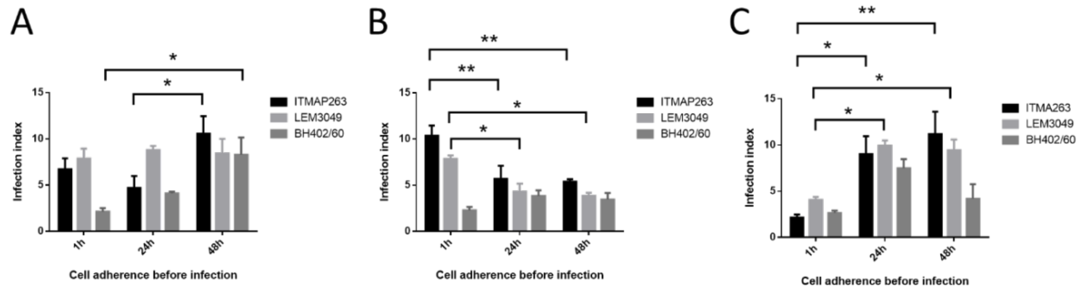

477

478 **Figure 2: Effect of cell adherence time on the infection index in peritoneal exudate cells (PECs) for Swiss mice (A),**
479 **BALB/c mice (B) and C57BL/6 mice (C).** Mice were stimulated with starch 24h before macrophage collection.
480 Cells were either left to adhere 1h, 24h or 48h prior to parasite infection. The infection indices were determined
481 24h post infection. Results are expressed as mean ± standard error of mean (SEM) and are based on two
482 independent replicate runs in duplicate (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

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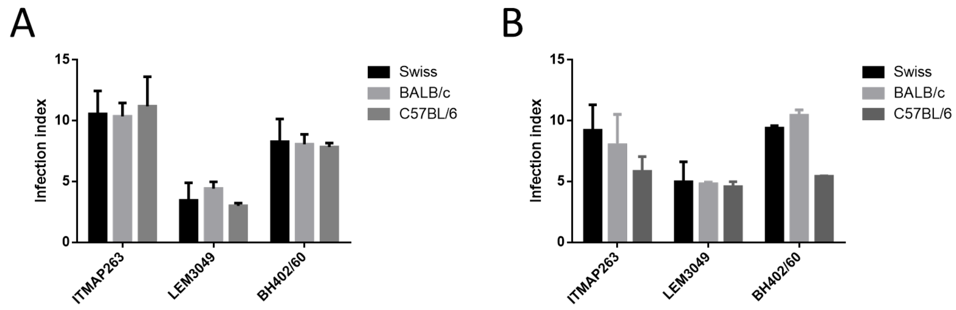

485

486 **Figure 3: Comparison of infection index at 24hpi using peritoneal exudate cells (PECs) from Swiss, BALB/c and C57BL/6**
487 **mice under optimal conditions of starch stimulation and cell adherence (strain-dependent) (A) or bone**
488 **marrow-derived macrophages (B).** Results are expressed as mean ± standard error of mean (SEM) and are
489 based on two independent replicate runs in duplicate (* $p<0.05$).

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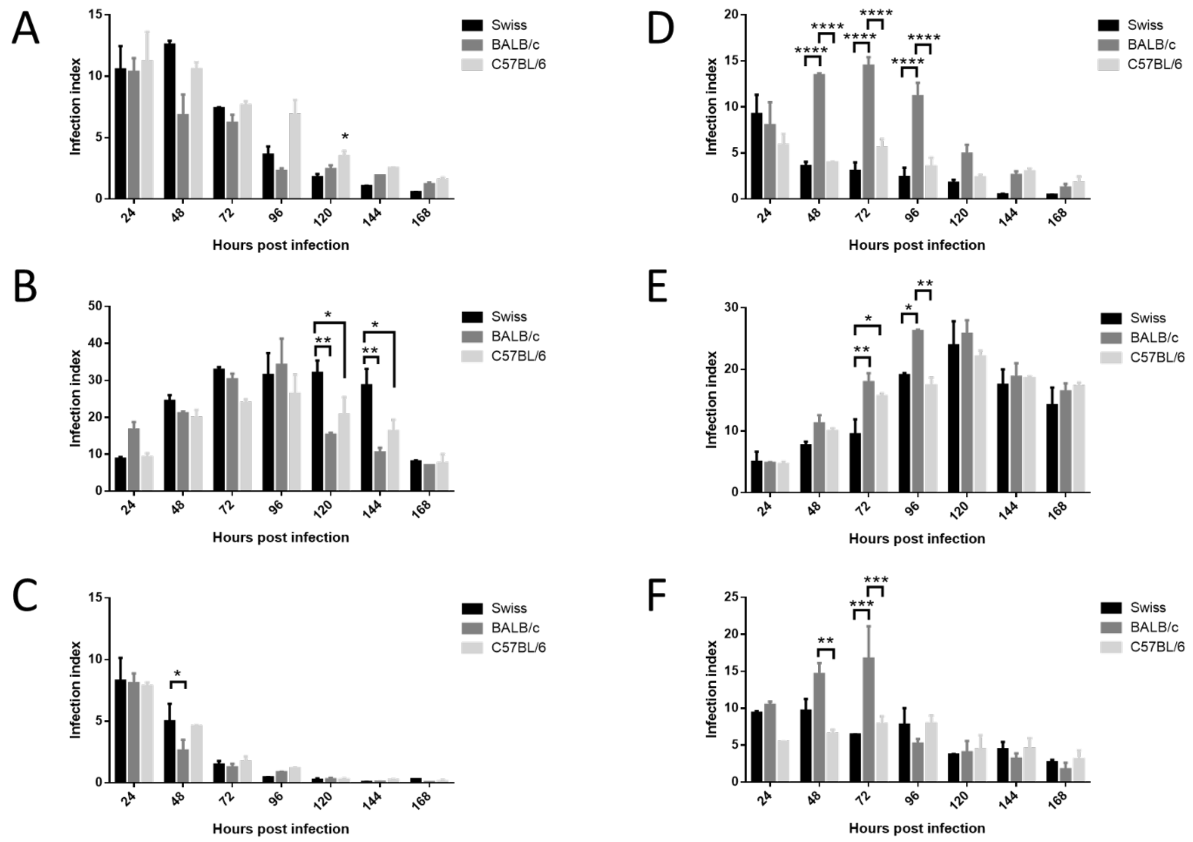

492

493 **Figure 4: Amastigote multiplication of the laboratory strain ITMAP263 (A+D) and the clinical isolates LEM3049 (B+E)**
494 **and BH402/60 (C+F) in peritoneal exudate cells (PECs) (left) or bone marrow-derived macrophages (BMMϕ)**
495 **(right) isolated from Swiss, BALB/c and C57BL/6 mice.** For each strain, PECs were used with the established
496 optimal conditions of starch stimulation and cell adherence. The average infection index is expressed from 24h
497 up to 168hpi. Results are expressed as mean ± standard error of mean (SEM) and are based on two independent
498 replicate runs in duplicate (* p<0.05; ** p<0.01; *** p<0.001).

499

500

501

502 **Figure 5: Percentage ROS production in BALB/c (A) and C57BL/6 (B) after different adherence time prior to infection**
503 **with the laboratory strain (ITMAP263) relative to the 2h of adherence time period (100%).** Results are
504 expressed as mean ± standard error of mean (SEM) and are based on two independent replicate runs in
505 duplicate (* $p<0.05$).

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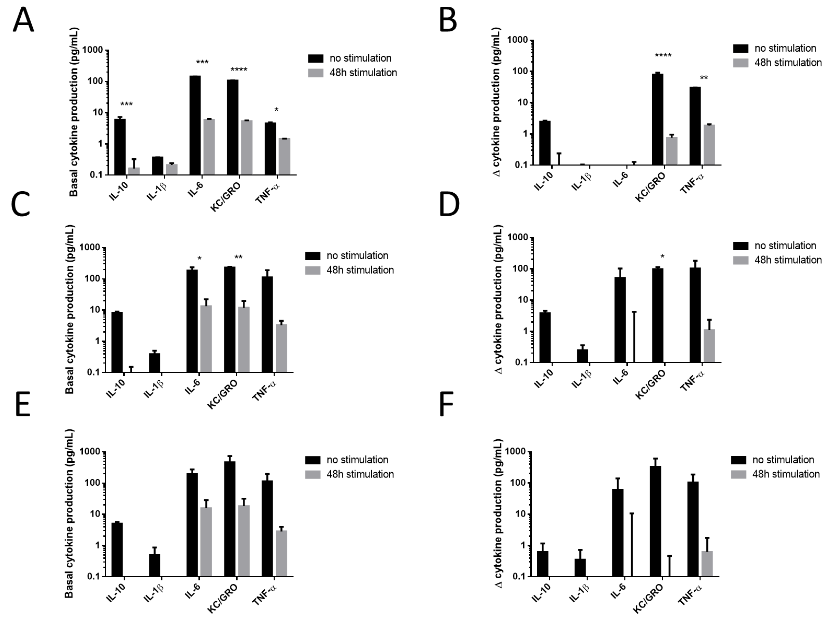

507

508 **Figure 6: Comparison of the basal cytokine production and the induced cytokine production between starch-stimulated**
509 **and naive Swiss PECs upon infection with the laboratory *Leishmania* strain ITMAP263 (A+B), or the clinical**
510 **isolates BH402/60 (C+D) and LEM3049 (E+F).** Results are expressed as mean ± standard error of mean (SEM)
511 and are based on two independent replicate runs in duplicate (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; ****
512 $p<0.0001$).

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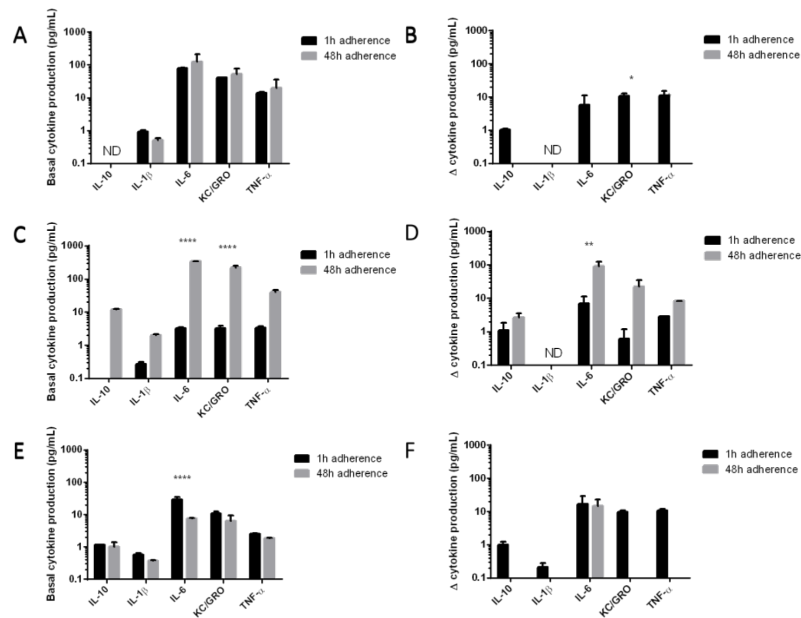
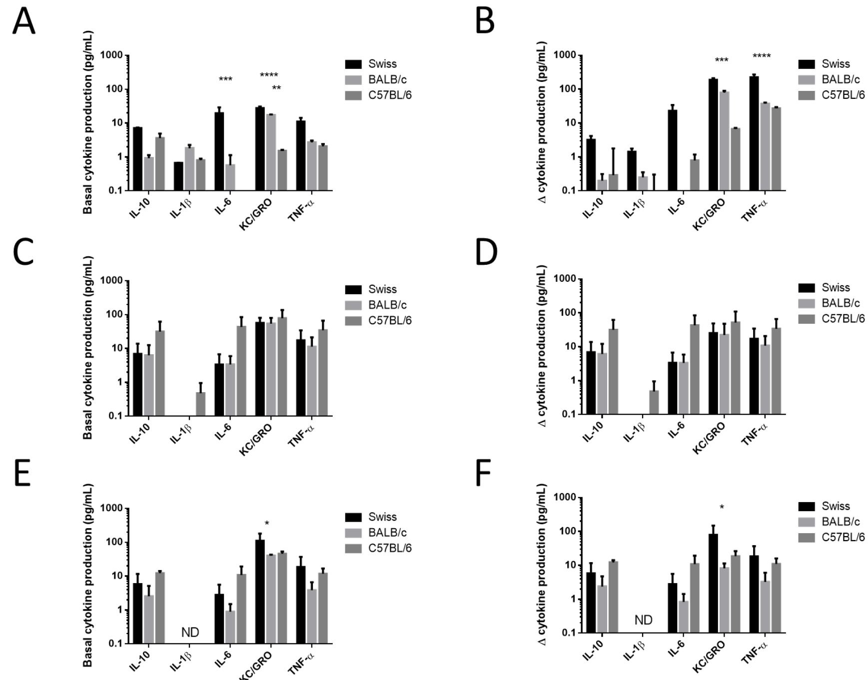


515

516 **Figure 7: Comparison of the basal (A,C,E) and the infection (ITMAP263)-induced (B,D,F) cytokine production between**
517 **1h-adhered or 48h-adhered PECs derived from Swiss (A+B), BALB/c (C+D) and C57BL/6 mice (E+F).** Results are
518 expressed as mean ± standard error of mean (SEM) and are based on two independent replicate runs in
519 duplicate (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$). ND: not detectable. Similar results were obtained
520 with two *L. infantum* clinical isolates (Figures S1 - S2).

521



522

523 **Figure 8: Comparison of the basal and the induced cytokine production between BMMϕ derived from Swiss, BALB/c or**
524 **C57BL/6 mice upon infection with the laboratory *Leishmania* strain (A+B) or the clinical isolates BH402/60**
525 **(C+D) and LEM3049 (E+F).** Results are expressed as mean ± standard error of mean (SEM) and are based on two
526 independent replicate runs in duplicate (* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$). ND: not detectable

527