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


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**Equal contribution

Running title – Primary macrophages for *Leishmania* infection *in vitro*

Keywords: *Leishmania*; host cell; primary macrophage;

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ABSTRACT

Primary mouse macrophages are frequently used to provide an in vitro intracellular model to evaluate antileishmanial drug efficacy. The present study compared the phenotypic characteristics of Swiss, BALB/c and C57BL/6 mouse bone marrow-derived macrophages and peritoneal exudate cells using different stimulation and adherence protocols upon infection with a Leishmania infantum laboratory strain and two clinical isolates. Evaluation parameters were susceptibility to infection, permissiveness to amastigote multiplication and impact on drug efficacy. Observed variations in infection of peritoneal exudate cells can mostly be linked to changes in the inflammatory cytokine profiles (IL-6, TNF-α, KC/GRO) rather than to differences in initial production of nitric oxide and reactive oxygen species. Optimization of the cell stimulation and adherence conditions resulted in comparable infection indices amongst peritoneal exudate cells and the various types of bone marrow-derived macrophages. Noteworthy is that BALB/c-derived bone marrow-derived macrophages were slightly more permissive to intracellular amastigote replication. Evaluation of antileishmanial drug potency in the various cell systems revealed minimal variation for antimonials and paromomycin, but no differences for miltefosine and amphotericin B. The study results allows to conclude that drug evaluation can be performed in all tested primary macrophages as only marginal differences were observed in terms of susceptibility to infection and impact of drug exposure.

Combined with some practical considerations, the use of 24h starch-stimulated, 48h-adhered, Swiss-derived peritoneal exudate cells can be advocated as an efficient, reliable, relatively quick and cost-effective tool for routine drug susceptibility testing in vitro whenever the use of primary cells is feasible.
**Introduction**

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

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

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

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

Collection of peritoneal macrophages: Primary peritoneal exudate cells (PECs) were collected from female Swiss, BALB/c and C57BL/6 mice, as described previously. Since most studies with PECs use either starch or thioglycolate stimulation to attract macrophages to the abdominal cavity and increase overall yield, different stimulation periods were compared. Mice were stimulated with a 2%
starch suspension in PBS either 48h or 24h prior to macrophage collection. To evaluate the phenotypic and immunological effects of the stimulation process, macrophages derived from non-stimulated mice were included as controls. Thioglycolate stimulation was not considered here as previous studies already demonstrated significant alterations of physiological macrophage properties \[^{[14]}\]. After collection, macrophages were counted and plated in 96-well plates at a concentration of 30,000 cells/well for the phenotypic evaluation. For NO measurements, cells were seeded at 800,000 cells/well while for the detection of ROS a concentration of \(10^6\) cells per well was adopted in a 24 well plate. The different types of PECs were incubated at 37°C in presence of 5% CO₂ and were allowed to adhere for 1h, 24h or 48h before infection with metacyclic promastigotes.

**Collection of bone marrow-derived macrophages:** Bone marrow-derived macrophages (BMMφs) were obtained from the femur and tibia of female Swiss, BALB/c and C57BL/6 mice by flushing the bone cavities with cold RPMI-1640 medium. The bone marrow was kept on ice during the isolation, then centrifuged for 20 minutes upon which the red blood cells were lysed with an ammonium-chloride-potassium lysis buffer (VWR, Leuven, Belgium ). The recovered cells were resuspended in RPMI-1640 medium supplemented with 1% non-essential amino acids, 1% penicillin-streptomycin solution, 1% sodium pyruvate, 1% L-glutamine, 10% iFBS and 15% L929 supernatant containing macrophage colony stimulating factor (M-CSF), and incubated in Petri dishes at 37°C and 5% CO₂ for 7 days in order to obtain macrophage monolayers. The cells were detached from the dishes with dissociation buffer (1% 0.5M EDTA, 2% 1M HEPES in PBS) 24h before infection, counted in KOVA® counting slides and seeded in 96-well plates in RPMI-1640 medium supplemented with 5% bovine serum, 2% penicillin-streptomycin and 1% L-glutamine at final concentrations similar to those used for the PECs.

**Receptivity for infection and intracellular amastigote multiplication:** Macrophages were infected with metacyclic promastigotes at a ratio of 10:1. For each condition, the intracellular parasite burden was quantified microscopically in at least of 50 macrophages, from which the infection index (average number of amastigotes/cell) was calculated \[^{[15]}\]. Infection indices at 24h post-infection (hpi) were
used to compare susceptibility to infection of the different macrophage host cells. The permissive capacity for infection was evaluated by assessing intracellular parasite replication over time, as described previously (16). In brief, intracellular amastigote burdens were assessed microscopically every 24h up to 168 hpi following methanol fixation and Giemsa-staining. As the initial receptivity for infection generally aligned well with the degree of intracellular amastigote replication, the 24hpi infection index was used to define the optimal conditions for the in vitro model.

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

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

Measurement of NO production: A Griess reagent kit (Promega G2930) was used for the determination of extracellular nitrite in infected macrophages. To avoid interference, RPMI-1640 without phenol red was used. After 24h of attachment, the cells were infected with preconditioned metacyclic promastigotes at an infection ratio of 10:1. After 48h of incubation, the supernatant was transferred to a 96-well plate (150µL/well) with the addition of 20 µL Griess reagent and 130 µL demineralized water. Samples were incubated for 30 min in the dark and absorbance was measured at 550nm (Labsystems Multiskan MCC/340). A standard calibration curve was set up by diluting the nitrite standard of the kit.

Cytokine production: To assess whether immunological differences between the various cell types may underline the observed phenotypic variations, the production of a cytokine subset with a known involvement in active leishmaniasis was evaluated by multiplex ELISA. Supernatants of the different cell types were collected 48h after infection and kept at -80°C until analysis. Cytokines were quantified in a V-plex custom mouse cytokine plate (IL-1β, IL-6, KC/GRO, TNF-α and IL-10) using the multiplex electro-chemiluminescent detection Quickplex SQ120 system (Meso Scale Discovery) following to the manufacturer’s instructions.

Statistical analysis: All statistical analyses were performed using Graphpad Prism version 4.00 software. Statistical differences between the different macrophage types and between the different conditions within one group were determined using 2-way ANOVA with Bonferroni post-hoc comparisons. Intergroup comparisons were done using non-parametric Friedman test followed by Dunn's post-hoc comparisons. Tests were considered statistically significant if p <0.05.
**Results**

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

The susceptibility of PECs from the various mouse genetic backgrounds (Swiss, BALB/c and C57BL/6) to *L. infantum* infection and the impact of starch stimulation and cell adherence time was based on the mean number of intracellular amastigotes at 24hpi for the different PEC types. For all tested parasite strains, the highest infection indices were reached in the starch stimulated PECs, most clearly in PECs derived from Swiss and C57BL/6, while BALB/c PECs showed an intrinsically higher infection susceptibility without a significant benefit from the stimulation (Figure 1).

The impact of cell adhesion time prior to infection (1h, 24h or 48h) on macrophage infection was also evaluated at 24hpi. For all tested strains, infection indices increased with the adherence time of Swiss and C57BL/6 PECs (Figure 2). As for the starch stimulation, prolonged adherence of the intrinsically more susceptible BALB/c PECs did not enhance but rather decreased parasite infection burdens. In summary, the highest infection indices were reached using the optimal combination of 24h starch stimulation and 48h cell adherence for both Swiss- and C57BL/6-derived PECs. The highest infection burdens in BALB/c-derived PECs were achieved with 24h starch stimulation and only 1h cell adhesion. PECs collected according to these optimized conditions and bone marrow-derived macrophages were subsequently compared for overall susceptibility to infection and amastigote multiplication.

**Impact of cell source on infection and intracellular amastigote multiplication**

PECs isolated under the optimal conditions of starch stimulation and cell adherence for each strain did not reveal significant differences in the infection indices (Figure 3A). When comparing the different BMMφ with the PECs, no statistical differences could be detected. C57BL/6 BMMφ seemed slightly less susceptible to infection (Figure 3B).
Although marginal differences could be observed between the PECs from the various mouse strains, the overall intracellular amastigote replication profiles were fully comparable (Figure 4A+B+C). The clinical LEM3049 strain was clearly showing intracellular amplification in PECs as compared to the limited or no expansion of the ITMAP263 and BH402/60 strains. Swiss and C57BL/6-BMMφ harbored fully comparable parasite burdens. Remarkably, BALB/c-derived BMMφ supported heavier amastigote replication, even for the strains that did not expand significantly in PECs (Figure 4D+E+F).

Immunological observations correlate with the observed differences in infectivity

ROS, NO and cytokine production were assessed in relation to the observed differences in cell infectivity and intracellular amastigote proliferation. The increased susceptibility to infection upon prolonged adherence of C57BL/6 PECs correlated with significantly lower ROS production (Figure 5), whereas ROS levels in PECs of BALB/c and Swiss mice remained unaltered. No statistical differences could be detected in ROS production of BMMφ originating from the various mouse strains. No biologically relevant changes in NO production were observed in both PECs and BMMφ of Swiss, BALB/c and C57BL/6 mice.

The pronounced increase in susceptibility of starch stimulated Swiss PECs could be linked to overall lower basal levels of the tested cytokines and a less prominent TNF-α and KC/GRO response to infection (Figure 6). In C57BL/6 PECs, the increased parasite burdens upon prolonged adherence correlated with low infection-triggered TNF-α and KC/GRO production (Figure 7, Figures S1 - S2). Prolonged adherence of BALB/c-derived PECs provoked elevated basal and infection-induced cytokine levels corresponding with the observed lower infection indices.

Despite the clear difference in amastigote multiplication in BALB/c as compared to Swiss and C57BL/6 BMMφ, no differential cytokine response profile could be associated with this phenomenon (Figure 8).
Impact of cell source on antiparasitic drug susceptibility profiling

The drug susceptibility (IC\textsubscript{50}-values) of intracellular amastigotes for the panel of antileishmanial reference drugs (Sb\textsuperscript{V}, Sb\textsuperscript{III}, MIL, PMM and AmB) was examined for each cell type (Table 1). Small shifts in susceptibility depending on the different starch stimulation periods and cell adherence times can be observed. Although drug susceptibility for Sb\textsuperscript{III}, MIL and AmB did not significantly vary between the various PECs, some differences could be observed for Sb\textsuperscript{V} and PMM. The Sb\textsuperscript{V} susceptibility in Swiss- and BALB/c-derived PECs decreased as a result of starch induction, which actually coincides with higher intrinsic parasite burdens. Similarly, the higher infection indices in BALB/c PECs following shorter cell adherence times coincided with decreased drug susceptibility. Some variation was observed for PMM which mostly related to the starch stimulation. In general, \textit{L. infantum} amastigotes were found to be more resistant to Sb\textsuperscript{V} and Sb\textsuperscript{III} in BMMϕ than in PECs. The higher intracellular amastigote burdens in BALB/c BMMϕ did not result in major changes in the drug efficacy results, but correlated to the relatively lower IC\textsubscript{50} value for PMM as compared to the one obtained in PECs (p<0.05).
Various host cell types have been used for in vitro drug susceptibility evaluation of *Leishmania*. Although some laboratories may have no access to primary cells and remain restricted to the use of commercially available cell lines, it is generally well-accepted that primary macrophages provide a more optimal and robust intracellular model that has advantages in terms of biological relevance.

Research comparing primary peritoneal mouse macrophages (PECs), mouse bone marrow-derived macrophages (BMMφ) and human blood monocyte-derived macrophages (PBMCs) already indicated differences in drug susceptibility dependent on the host cell type \(^{(10)}\). Despite these findings, different cell types are still being widely used for drug screening and susceptibility evaluation, highlighting a need for assay harmonization. Furthermore, the collection of primary macrophages can be performed adopting diverse procedures, including various mouse strains and following different stimulation and cell adherence protocols. The present study on primary macrophages specifically focused on comparing the phenotypic and immunological consequences of these variables on promastigote-induced infection, support of intracellular parasite expansion and drug susceptibility.

Firstly, the production of superoxide (O\(_2^−\)) and nitric oxide (NO) upon parasite infection was evaluated as these are known key factors in the establishment of infection. The initial increase in O\(_2^−\) occurring when promastigotes are phagocytosed by macrophages \(^{(17)}\) has been shown to be toxic for promastigotes \(^{(18, 19)}\), while NO as a second oxidant is mostly effective in killing the intracellular parasite generally after macrophage activation by IFN-γ and TNFα \(^{(20-22)}\). Although the parasite has developed several defense strategies to overcome the detrimental effects of O\(_2^−\) and NO, such as intracellular thiols and T(SH)\(_2\) \(^{(23, 24)}\), some of the current antileishmanial reference drugs have been shown to counteract these defense mechanisms and increase the intracellular NO and/or O\(_2^−\) production \(^{(25-27)}\). Recognizing the pivotal role of NO and O\(_2^−\), using an intracellular in vitro model where both are produced in situ appears imperative for appropriate drug susceptibility assays. Cell adherence was also found to be an important parameter determining the susceptibility of PECs to *L. infantum* infection. NO and O\(_2^−\) measurements only revealed a decreased ROS production in C57BL/6
PECs upon prolonged adherence and was linked to an increased susceptibility to infection. Starch stimulation induced higher infections in Swiss PECs, but could not be linked to biologically relevant differences in ROS and NO production. The observation of a markedly increased intracellular parasite survival in BALB/c BMMφ corroborated earlier studies linking host immunology to the cell’s phenotype in vitro for other pathogens \(^{[2, 3, 28]}\), but was not reflected by a lower NO or O\(_2^\cdot\) production in the present study.

Secondly, several cytokines have already been implicated in driving the infection dynamics of *Leishmania in vivo* \(^{[29, 30]}\). In animal models, the disease outcome is shown to be determined by a delicate innate balance between several pro- and anti-inflammatory cytokines \(^{[31]}\). Numerous studies investigated the immunological responses of macrophages to *L. major* infection, with only limited induction of gene expression \(^{[32]}\). Earlier research reported the induction of several pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 upon phagocytosis of *Leishmania* parasites \(^{[33-35]}\), while the production of anti-inflammatory cytokines such as IL-10 has been described as well \(^{[36]}\). Although IL-10 is a known suppressor of inflammatory cytokine production, thereby exacerbating disease progression, its production upon parasite infection in this *in vitro* study was minimal and is in agreement with previous research \(^{[34, 37]}\). Past research also linked the production of IL-10 to a decreased NO production and reduced parasite killing \(^{[38]}\), but no correlation with the observed differences in macrophage infection could be demonstrated with the VL strains used in the present study. The increasing infection rates upon starch-stimulation of the Swiss PECs and upon longer cell adherence of Swiss- and C57BL/6-derived PECs was found to be linked to a decreased pro-inflammatory KC/GRO and TNF expression profile, known to regulate the control of *Leishmania* infection \(^{[33-35]}\). KC/GRO is the CXCL1 chemokine that contributes *in vivo* to the recruitment of macrophages and neutrophils to the site of infection \(^{[32]}\) and TNF is a known inducer of leishmanicidal activity in the macrophage. Beside a lower KC/GRO and TNF responsiveness, the beneficial effect of starch stimulation on parasite receptivity of Swiss PECs was also linked to a decreased basal activation status, *e.g.* overall lower levels of IL-10, IL-6, KC/GRO and TNF-α. It is assumed that the
observed differences in cytokine profile are associated with various other phenomena such as membrane trafficking, the parasite's access to nutrients, apoptosis or autophagy that all may affect Leishmania growth (39-41). In addition to increasing the infection ratio upon infection, starch stimulation also resulted in a higher cell yield during peritoneal macrophage collection hereby significantly diminishing the number of laboratory animals needed. The findings obtained with the reference laboratory strain ITMAP263 were validated for the recent L. infantum clinical isolates LEM3049 and BH402/60. Overall, the main conclusions were comparable for all strains. Slight inter-strain differences, however, indicate that starch stimulation and cell adherence conditions could require some degree of individual fine-tuning when aiming to produce maximal infection burdens. Next to the observed inter-strain differences, an impact of the infecting parasite-stage on infectivity and intracellular infection dynamics can be anticipated as well. While this study used metacyclic promastigotes for infection, conditions of starch stimulation and cell adherence might require some modifications when working with ex vivo-derived amastigotes. Although the phenotypic and immunological implications of the adopted protocols are important criteria to select the optimal conditions for in vitro drug susceptibility evaluation, ensuing practical issues should be considered as well. Collection of BMMφ is complex, requires more steps and is more time-consuming because bone marrow-progenitor cells should be differentiated into macrophages by addition of M-CSF to the culture medium (42). BALB/c BMMφ appeared to be the most susceptible cell type for supporting intracellular amastigote amplification of all parasite strains tested. In contrast to the tedious process of obtaining BMMφ, the collection of high numbers of macrophages from the peritoneal cavity is far more straightforward and will reduce the number of required laboratory animals, a highly relevant factor when responsibly conducting animal experiments. Although the activation state of the macrophages may influence drug susceptibility, the in vitro drug susceptibility values obtained in the various cell systems were quite comparable (Table 1). The recorded decrease in susceptibility to SbV with increasing parasite burdens is not unexpected given that similar 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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<table>
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<th>Cell conditioning</th>
<th>Sb&lt;sup&gt;v&lt;/sup&gt; (eq.)</th>
<th>Sb&lt;sup&gt;iii&lt;/sup&gt; (eq.)</th>
<th>Miltefosine (µM)</th>
<th>Paromomycin (µM)</th>
<th>Fungizone&lt;sup&gt;®&lt;/sup&gt; (µM)</th>
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<td>48h stimulation</td>
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<td>64.8 ± 22.3</td>
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<td>48h stimulation</td>
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<td>3.9 ± 0.7</td>
<td>0.5 ± 0.0</td>
<td>40.5 ± 5.5</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>24h stimulation</td>
<td>42.5 ± 11.9</td>
<td>3.5 ± 0.6</td>
<td>0.4 ± 0.1</td>
<td>47.2 ± 8.1</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>no stimulation</td>
<td>24.8 ± 0.6</td>
<td>2.7 ± 0.4</td>
<td>0.5 ± 0.0</td>
<td>72.2 ± 7.8</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td><strong>1h adherence</strong></td>
<td></td>
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<tr>
<td>48h stimulation</td>
<td>77.0 ± 0.0</td>
<td>8.3 ± 3.0</td>
<td>0.6 ± 0.1</td>
<td>66.0 ± 7.5</td>
<td>0.04 ± 0.00</td>
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<tr>
<td>24h stimulation</td>
<td>77.0 ± 0.0</td>
<td>5.3 ± 1.3</td>
<td>0.5 ± 0.0</td>
<td>73.3 ± 20.0</td>
<td>0.07 ± 0.02</td>
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<tr>
<td>no stimulation</td>
<td>72.8 ± 4.2</td>
<td>5.3 ± 1.7</td>
<td>0.5 ± 0.0</td>
<td>67.9 ± 11.1</td>
<td>0.04 ± 0.00</td>
</tr>
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<td><strong>48h adherence</strong></td>
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<tr>
<td>48h stimulation</td>
<td>31.5 ± 14.3</td>
<td>2.5 ± 0.5</td>
<td>0.7 ± 0.2</td>
<td>44.3 ± 4.3</td>
<td>0.02 ± 0.01</td>
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<tr>
<td>24h stimulation</td>
<td>46.4 ± 30.6</td>
<td>4.7 ± 1.3</td>
<td>0.7 ± 0.1</td>
<td>57.0 ± 10.0</td>
<td>0.04 ± 0.00</td>
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<tr>
<td>no stimulation</td>
<td>16.2 ± 14.4</td>
<td>7.9 ± 4.2</td>
<td>0.6 ± 0.1</td>
<td>59.2 ± 23.1</td>
<td>0.06 ± 0.03</td>
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<td><strong>24h adherence</strong></td>
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<tr>
<td>48h stimulation</td>
<td>68.4 ± 8.6</td>
<td>8.3 ± 2.7</td>
<td>0.8 ± 0.2</td>
<td>172.2 ± 69.9</td>
<td>0.03 ± 0.01</td>
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<tr>
<td>24h stimulation</td>
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<td>8.5 ± 3.1</td>
<td>0.7 ± 0.1</td>
<td>96.6 ± 24.0</td>
<td>0.04 ± 0.00</td>
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<tr>
<td>no stimulation</td>
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<td>2.0 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>107.1 ± 24.7</td>
<td>0.06 ± 0.00</td>
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<tr>
<td><strong>1h adherence</strong></td>
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<tr>
<td>48h stimulation</td>
<td>77.0 ± 0.0</td>
<td>6.8 ± 1.7</td>
<td>0.8 ± 0.2</td>
<td>54.0 ± 1.4</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>24h stimulation</td>
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<td>6.2 ± 1.5</td>
<td>0.7 ± 0.2</td>
<td>99.7 ± 35.4</td>
<td>0.06 ± 0.02</td>
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<td>0.5 ± 0.0</td>
<td>45.0 ± 13.2</td>
<td>0.04 ± 0.01</td>
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<td><strong>Bone marrow-derived mφ</strong></td>
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<tr>
<td>Swiss</td>
<td>77.0 ± 0.0</td>
<td>8.8 ± 0.5</td>
<td>0.9 ± 0.0</td>
<td>88.3 ± 34.1</td>
<td>0.07 ± 0.02</td>
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<tr>
<td>BALB/c</td>
<td>77.0 ± 0.0</td>
<td>11.1 ± 1.5</td>
<td>0.5 ± 0.1</td>
<td>25.3 ± 7.0</td>
<td>0.05 ± 0.02</td>
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<tr>
<td>C57BL/6</td>
<td>77.0 ± 0.0</td>
<td>10.9 ± 1.8</td>
<td>0.4 ± 0.1</td>
<td>60.6 ± 5.9</td>
<td>0.07 ± 0.03</td>
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</table>
Table 1: Drug susceptibility values (IC₅₀) against antileishmanial reference drugs for each cell type.

Cells were infected with the laboratory L. infantum strain ITMAP263. Results are expressed as the mean IC₅₀ ± standard error of mean (SEM) and are based on two independent replicates run in duplicate. Results (shaded) correspond to the starch stimulation and adherence conditions resulting in the highest parasite burdens.
Figure 1: Effect of starch stimulation on the infection index in peritoneal exudate cells (PECs) from Swiss (A), BALB/c (B) and C57BL/6 mice (C). Mice were either left unstimulated (no) or were stimulated with 2% starch 24h or 48h prior to collection of PECs. The cells were allowed to adhere 24h before infection. The infection indices were determined 24h post infection. Results are expressed as mean ± standard error of mean (SEM) and are based on two independent replicate runs in duplicate (* p<0.05; *** p<0.001).
Figure 2: Effect of cell adherence time on the infection index in peritoneal exudate cells (PECs) for Swiss mice (A), BALB/c mice (B) and C57BL/6 mice (C). Mice were stimulated with starch 24h before macrophage collection. Cells were either left to adhere 1h, 24h or 48h prior to parasite infection. The infection indices were determined 24h post infection. Results are expressed as mean ± standard error of mean (SEM) and are based on two independent replicate runs in duplicate (* p<0.05; ** p<0.01; *** p<0.001).
Figure 3: Comparison of infection index at 24hpi using peritoneal exudate cells (PECs) from Swiss, BALB/c and C57BL/6 mice under optimal conditions of starch stimulation and cell adherence (strain-dependent) (A) or bone marrow-derived macrophages (B). Results are expressed as mean ± standard error of mean (SEM) and are based on two independent replicate runs in duplicate (* p<0.05).
Figure 4: Amastigote multiplication of the laboratory strain ITMAP263 (A+D) and the clinical isolates LEM3049 (B+E) and BH402/60 (C+F) in peritoneal exudate cells (PECs) (left) or bone marrow-derived macrophages (BMMφ) (right) isolated from Swiss, BALB/c and C57BL/6 mice. For each strain, PECs were used with the established optimal conditions of starch stimulation and cell adherence. The average infection index is expressed from 24h up to 168hpi. Results are expressed as mean ± standard error of mean (SEM) and are based on two independent replicate runs in duplicate (* p<0.05; ** p<0.01; *** p<0.001).
Figure 5: Percentage ROS production in BALB/c (A) and C57BL/6 (B) after different adherence time prior to infection with the laboratory strain (ITMAP263) relative to the 2h of adherence time period (100%). Results are expressed as mean ± standard error of mean (SEM) and are based on two independent replicate runs in duplicate (* p<0.05).
Figure 6: Comparison of the basal cytokine production and the induced cytokine production between starch-stimulated and naive Swiss PECs upon infection with the laboratory *Leishmania* strain ITMAP263 (A+B), or the clinical isolates BH402/60 (C+D) and LEM3049 (E+F). Results are expressed as mean ± standard error of mean (SEM) and are based on two independent replicate runs in duplicate (* p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001).
Figure 7: Comparison of the basal (A,C,E) and the infection (ITMAP263)-induced (B,D,F) cytokine production between 1h-adhered or 48h-adhered PECs derived from Swiss (A+B), BALB/c (C+D) and C57BL/6 mice (E+F). Results are expressed as mean ± standard error of mean (SEM) and are based on two independent replicate runs in duplicate (* p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001). ND: not detectable. Similar results were obtained with two L. infantum clinical isolates (Figures S1 - S2).
Figure 8: Comparison of the basal and the induced cytokine production between BMMΦ derived from Swiss, BALB/c or C57BL/6 mice upon infection with the laboratory *Leishmania* strain (A+B) or the clinical isolates BH402/60 (C+D) and LEM3049 (E+F). Results are expressed as mean ± standard error of mean (SEM) and are based on two independent replicate runs in duplicate (* p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001). ND: not detectable.