A further investigation of the leishmaniosis outbreak in Madrid (Spain): low-infectivity phenotype of the *Leishmania infantum* BOS1FL1 isolate to establish infection in canine cells.

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Abstract

Human leishmaniosis caused by *Leishmania infantum* is a zoonotic disease, with dogs as the main reservoir in Mediterranean Basin countries. The largest European outbreak of human leishmaniosis declared in the southwestern Madrid region (Spain) is characterized by unusual epidemiological and clinical features, such as the emergence of new wild reservoirs (hares and rabbits), whereas the seroprevalence, infection, and severity of canine leishmaniosis have not substantially changed since the first studies conducted in Madrid before the outbreak. Previous studies reported that *L. infantum* isolates from the Madrid leishmaniosis focus displayed elevated virulence in *in vivo* models of infection and increased infectivity in murine target cells. With the aim of studying whether changes in the host-parasite interaction and virulence profile have developed, we first assessed the behaviour of one circulating isolate of the outbreak, IPER/ES/2012/BOS1FL1 (BOS1FL1), compared to that of a well-characterized strain from canine leishmaniosis, MCAN/ES/1996/BCN150 (BCN150), in terms of infection capacity (percentage of infected cells, representing infectivity, and number of amastigotes per infected cell, representing the intensity of infection) in canine monocytes and macrophages. BCN150 displayed significantly higher infectivity (76.82 ± 4.40 *vs* 38.58 ± 2.19; $P < 0.0001$) and intensity of infection (3.64 ± 0.13 *vs* 1.83 ± 0.12; $P < 0.0001$) than BOS1FL1 when interacting with canine cells. Our ROS induction results did not differ significantly between the two isolates or with the responses previously described for other *L. infantum* isolates. Paradoxically, increased resilience to hydrogen peroxide exposure was observed for BOS1FL1 (% viability 40.62 ± 5.54 *vs* 26.37 ± 2.93; $P = 0.039$). Finally, we demonstrated that a decreased intracellular load of BOS1FL1 was associated with increased IFN-γ (261.21 ± 26.29 *vs* 69.80 ± 9.02; $P = 0.0151$) and decreased IL-10 production (165.06 ± 23.87 *vs* 264.41 ± 30.58; $P = 0.0002$). In this study, we provide the

first detailed insight into the differences between the isolate BOS1FL1 from the outbreak in Madrid and the well-characterized strain BCN150 MON-1 obtained from a dog in their response to interacting with canine cells. However, further studies are necessary to shed light on the immune mechanisms resulting in BOS1FL1 exhibiting less virulent behaviour in canine cells than in cells derived from other host species.

Key Words: *Leishmania infantum*, ROS, Monocytes, Dogs, Virulence, Tropism

1. Introduction

Visceral leishmaniosis (VL) (Kassai et al., 1988) in the Mediterranean basin is a protozoan zoonotic disease caused by *Leishmania infantum* and transmitted by *Phlebotomus* species of sandfly vectors to humans and other mammals. Dogs are considered the main domestic reservoir for *L. infantum* (Ashford, 1996). In Spain, canine leishmaniosis (CanL) is endemic, and human leishmaniosis (HumL) has significantly increased in incidence since 2009 due to the human leishmaniosis outbreak in the southwestern region of Madrid (Spain). This outbreak is the largest outbreak in Europe to date, with more than 700 cases (corresponding to a great increase in VL incidence (35.9%), with 64.1 % increase in cutaneous leishmaniosis (CL) incidence). Remarkably, 70% of the patients are immunocompetent and atypical localized lymphadenopathic leishmaniosis (LLL) has appeared in 19% of the VL cases (Arce et al., 2013; Horrillo et al., 2015). Even though the dog is the main reservoir in the Mediterranean basin, disease prevalence studies in dogs in the area did not reveal a significant increase in cases of CanL (Miro et al., 2017). In this context, xenodiagnosis studies, based on the exposure of infected lagomorphs to vector bites to assess their ability to transmit protozoa, have shown lagomorph species (hares and rabbits) to play a role as sylvatic reservoirs (Jimenez et al., 2014; Molina et al., 2012). Moreover, there are also molecular typing studies that conclude that the human leishmaniosis outbreak of Madrid is not the responsibility of a newly emerging genotype of *L. infantum*, since the parasitic isolates do not show significant differences in their genotype from the strains circulating in a traditional way in the cases of CanL in Madrid (Chicharro et al., 2013).

In the development of leishmaniosis, how the interaction between the parasite and the host immune response occurs is fundamental to the establishment and subsequent outcome of the disease (Scott and Novais, 2016). Immediately after infection, the innate immune response is activated in the host and is characterized by the mobilization of neutrophils and cells of the phagocytic mononuclear system (monocytes and macrophages) (Hurrell et al., 2016; Moreira et al., 2016). The way in which this interaction between monocytes and parasites occurs is critical to the susceptibility or resistance outcome of infection. In this regard, host cells have mechanisms of defence against infection, such as the development of the respiratory burst (RB) that is involved in the production of compounds with leishmanicidal activity, such as nitric oxide (NO) and reactive oxygen species (ROS) (Carneiro et al., 2016). NO production is dependent on the expression of the enzyme nitric oxide synthetase resulting from the activation of phagocytic cells by gamma-interferon (IFN-ɣ) during the development of a Th1-type response. The production of ROS is a purely innate mechanism triggered by phagocytosis of promastigotes. However, previous studies have shown that there are differences in the ability of phagocytic cells to produce both compounds, depending on the species of origin (Novais et al., 2014).

In a previous study, using an *ex vivo* murine model of VL, we revealed the high virulence of the *L. infantum* isolate circulating in the outbreak, IPER/ES/2012/BOS1FL1 (BOS1FL1), compared with that of the strain MCAN/ES/1996/BCN150 (BCN150), which was isolated from a dog (Fernandez-Cotrina et al., 2013). BOS1FL1 displays a high intensity of infection and compromises the integrity of the immune defence machinery of murine macrophages and dendritic cells (Dominguez-Bernal et al., 2014). Furthermore, strong virulence of isolates circulating in the focus has been demonstrated

in vivo in BALB/c and hamster models of VL, which exhibit elevated parasite loads in the spleen and liver accompanied by histological damage (Martin-Martin et al., 2015; Mas et al., 2020). Indeed, high *L. infantum* loads in the vector *Phlebotomus perniciosus* from the focus area have been found (Gonzalez et al., 2017). These facts support the exceptional features of this outbreak. In the context of the unusual eco-epidemiological properties of the leishmaniosis outbreak in Madrid, the aim of our study was to deepen the knowledge of the diversity of pathogen host tropism. Thus, we compared the *ex vivo* interactions of the *L. infantum* BOS1FL1 isolate with that of the BCN150 strain after infection of canine monocytes (canMon) and macrophages (canMø).

2. Materials and Methods

2.1. Ethics statement

All the processes described in this manuscript complied with the Spanish (Law 63/2013) and European Union legislation (2010/63/UE), and they were approved by the Ethics and Welfare Committee for Animal Experimentation of Complutense University of Madrid and Comunidad de Madrid authorities under procedural number PROEX 05/2018.

2.2. Parasites

The *L. infantum* IPER/ES/2012/BOS1FL1 (BOS1FL1) isolate was obtained from *P. perniciosus* captured in the leishmaniosis outbreak in the southwestern region of Madrid (Spain) (Martin-Martin et al., 2015), and it was compared to the well-characterized strain named MCAN/ES/1996/BCN150, zymodeme MON-1 (BCN150), which has been used previously in other studies related to canine visceral leishmaniosis (CanVL) (Fernandez-Cotrina et al., 2013). With the purpose of minimizing potential changes in virulence due

to prolonged maintenance under *in vitro* conditions, both *L. infantum* strains were passaged through BALB/c mice (*n* = 1 mouse/isolate) as described previously (Dominguez-Bernal et al., 2015). For re-isolation, two months after infection, spleens were homogenized and cultivated in NNN medium at 26 ºC for 4-7 days until promastigotes expanded (passage number 1). Then, they were grown in Schneider's medium (Dominique Dutscher, Issy-les-Moulineaux, France) supplemented with 20% heat-inactivated foetal bovine serum (FBS, Gibco, Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and a mixture of antibiotics (200 U/mL penicillin, 200 µg/mL streptomycin) (Lonza, Basel, Switzerland), and they were maintained at 26 ºC until the infection assays. Similar passages between isolates were compared, and no passage greater than 8/10 was employed for the studies.

2.3. *In vitro* growth kinetics and metacyclogenesis

Promastigotes of both isolates were centrifuged and resuspended in 4 mL of complete Schneider's medium with a final concentration of 5 x 10^5 parasites/mL. They were incubated at 26 ºC for 20 days without adding fresh medium until the concentration was below the initial concentration. Parasite densities were obtained by daily counting of viable promastigotes using a Neubauer chamber (Brand). Promastigote morphology was evaluated microscopically. The promastigote flagellum/cell body length ratio was determined as a measure for metacyclogenesis as described elsewhere (Ouakad et al., 2011). Parasites were considered metacyclic when presenting a ratio >2.

2.4. Hydrogen peroxide stress assay

To assess the inhibitory effect of reactive oxygen species on parasite growth, 10^7 promastigotes/mL stationary phase *L. infantum* BCN150 and BOS1FL1 were incubated

in a 96-well flat-bottomed plate in complete Schneider's medium supplemented with hydrogen peroxide (H_2O_2, Panreac, Castellar del Vallés, Spain) in increasing concentrations (0-10 mM). After 2 h at 26 ºC, MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Molecular Probes by Life Technologies] solution (5 mg/mL) was added and incubated at 37 ºC for 1 h. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) was used to dissolve the formazan crystals. Optical density was measured in a spectrophotometer at 570-660 nm. Triton™ X-100 (2%) (Sigma-Aldrich) was used as a negative control.

2.5. Isolation of canMon and canMø from peripheral blood

Healthy canine donors (*n* = 10) of different breeds, middle age and both sexes that complied with the criteria of being negative for *Leishmania* by serology and PCR were employed. A total of 250 mL of whole peripheral blood was collected via jugular venepuncture from each dog in citrate-phosphate-dextrose-adenine (CPDA) anticoagulant. Peripheral blood mononuclear cells (PBMCs) were isolated through density gradient centrifugation (Histopaque-1077, Sigma-Aldrich) as previously described (Araujo et al., 2011; Martínez-Rodrigo et al., 2019). Monocytes were obtained by magnetic separation using mouse anti-human CD14-coupled microbeads (Miltenyi Biotec Ltd., USA) according to the manufacturer's instructions and maintained in complete medium (CM) consisting of RPMI-1640 medium with 2 mM L-glutamine (GE Healthcare, Spain) supplemented with 10% FBS, a mixture of antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) (Lonza) and 10 mM HEPES (Lonza) at 37 ºC until infection. CD14-negative cells were collected and frozen in CM containing 40% FBS and 10% dimethyl sulfoxide (DMSO) and stored at -80 °C until the infection assay was performed. For macrophage differentiation, CD14-positive monocytes (6 x 106 cells per

mL) were seeded in 25 cm2 flasks and differentiated into canMø by the addition of 33 ng/mL recombinant human Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF, Kingfisher Biotech, Saint Paul, MN) for a period of 7 days at 37 °C and 5% CO2. At day 3, the medium was replaced with medium containing the same concentration of freshly added growth factor. To determine the phenotype of canMon and canMø, both cells were double stained on days 0 and 7 with αCD14 (mouse anti-human CD14:RPE, TÜK4, Bio-Rad, Madrid, Spain) in combination with αMHC-II (rat anti-dog MHC class II, YKIX334.2, Bio-Rad) and αCD86 (mouse anti-human CD86:RPE, BU63, Bio-Rad) in combination with αCD40 (mouse anti-human CD40:FITC, LOB7/6, Bio-Rad). CanMon were characterized on day 0 as CD14+ MHC class II+, CD40+/- and CD86- cells, while canMø were immunophenotyped on day 7 as CD14+/- MHC class II +/-, CD40+ and CD86- cells. CD14-negative cells were stained with αCD3 (rat anti-canine CD3, CA17.2A12, Bio-Rad) to characterize them as T lymphocytes (CD14-CD3+). Fluorescence intensities were quantified using a FACScan flow cytometer (BD Biosciences, CAI, UCM, Madrid, Spain), and data were processed with FlowJo X software version 10.0.7 (Beckton Dickinson, Franklin lakes, NJ, USA).

2.6. *L. infantum ex vivo* infection assays

Cells (canMon or canMø) were seeded at a concentration of $5x10^5$ cells on poly-L-lysine (Sigma Aldrich)-coated 13 mm diameter coverslips into P24 cell culture plates to improve cell adherence. After 12 h, cells were infected with stationary phase *L. infantum* promastigotes at a ratio of 5:1 (parasites:cells) at 37 ºC in 5% CO_2 for 4 h. Subsequently, extracellular parasites were removed by washing the cells, and they were incubated with autologous lymphocytes as a co-culture (5:1 ratio lymphocytes:monocytes/macrophages) in complete medium for an additional 24 and 72 h (Almeida et al., 2017). To determine

196 the antileishmanial activity of canMon and canMø, 400 cells were counted in duplicate
197 after Giemsa staining under an Olympus BX41 optical microscope. The percentage of
198 infected cells (referred to as infectivity) and the mean number of amastigotes per infected
199 cell (referred to as intensity of infection) were evaluated. Infection index was calculated
200 by multiplication of both parameters as previously described (Dominguez-Bernal et al.,
201 2014).
202
203 **2.7. Development of the oxidative burst**
204 NO production was evaluated indirectly by assessing the nitrite concentration using the
205 Griess reagent. Cells (canMon and canMø) were infected with *L. infantum* at a ratio of
206 5:1 parasites:cells for 24 and 72 h. After 4 h of infection, extracellular promastigotes were
207 removed by washing, and cells were stimulated with lipopolysaccharide (LPS) (1 µg/mL,
208 Sigma-Aldrich) (Ding et al., 1988; Dominguez-Bernal et al., 2012). ROS levels were
209 determined by flow cytometry using DHE (Dihydroethidium, Sigma-Aldrich). When
210 required, cells were pre-treated with PMA (Phorbol 12-myristate 13-acetate, Sigma-
211 Aldrich) at 1 µg/mL as an ROS inducer (Ponath and Kaina, 2017). Infected cells were
212 incubated with 10 mM DHE for 30 minutes at 37 ºC in a CO_2 incubator. PMA (1 µg/mL,
213 Sigma-Aldrich)-treated cells were used as a positive control (Wang et al., 2013).
214
215 **2.8. Quantification of cytokine, TLR-2, TLR-4, arginase and nitric oxide mRNA**
216 **expression levels**
217 Total RNA for cytokine expression analysis was extracted from the abovementioned
218 infected canMon- and canMø by an NZY Total RNA Isolation Kit (NZYTech, Lisbon,
219 Portugal) following the manufacturer's instructions. Reverse transcription was carried out
220 using an NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal). Between

200 and 400 ng of total RNA was analysed in each sample. Primers used for canine Arginase, NO synthetase, Toll-like receptors (TLR-2 and TLR-4), IL-1β, IL-6, IL-10, IL-12p40, IL-18, TNF-α, and the housekeeping genes β-actin and GAPDH were designed in this study and are listed in supplementary Table S1. Real-time PCRs were performed in 20 µL using NZYSpeedy qPCR Green Master Mix (2x) (NZYTech, Lisbon, Portugal), 5 pmol of each primer and 4 µL of diluted cDNA samples in a 7500 FAST Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative quantification of cytokine mRNA expression levels (fold change in expression) was carried out by the comparative $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

2.9. Quantification of cytokine production and arginase activity in the co-culture system

Cells (canMon and canMø) were cultured overnight in P24 tissue culture-treated plates. To determine cytokine production (TNF-α, IFN-ɤ and IL-10), canMon/canMø were infected with *L. infantum* and co-cultured with autologous lymphocytes as described previously. Culture supernatants were collected after 72 h, and cytokines were determined by ELISA according to the manufacturer's instructions (Duoset ELISA Development System Kits R&D Systems, Minneapolis, MN, USA). The intracellular arginase activity from infected cells was measured as previously described (Dominguez-Bernal et al., 2015) after incubating for 30 min in lysis buffer (0.1 M Tris-HCl, pH 7.5, 300 µM NaCl, 1 µM PMSF, 1% Triton X-100). One unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1 mMol of urea/min.

2.10. Statistical Analysis

Data sets were analysed as the mean ± standard error of the mean (SEM). The significance of the growth curve and MTT assay results was calculated using a parametric statistical test: Student's *t* test for pairwise comparisons. For the evaluation of ROS production, *ex vivo* infection assays and qPCR results, a parametric statistical test was used: one-way ANOVA followed by Tukey's test for multiple comparisons. The arginase activity, NO production and cytokine production (measured by ELISA) were analysed by two-way ANOVA followed by Tukey's test for multiple comparisons. All analyses were performed using GraphPad Prism software (version 6.0 for Windows, San Diego, CA, USA). Significant differences were determined and are designated with asterisks, as follows: $*P < 0.05$, $**P < 0.01$.

3. Results

3.1. *In vitro* characterization of *L. infantum* isolates

With the aim of ruling out that any different putative *in vivo* behaviour between the isolate BOS1FL1 and the reference strain (BCN150) was associated with a modification in growth features, we analysed the *in vitro* growth kinetics of promastigotes from both isolates over 20 days in culture medium. BCN150 and BOS1FL1 did not differ in their speed of growth to reach the stationary phase or the highest density of viable parasites at the same time between the 5^{th} and 8^{th} days (Fig. 1A). The development of metacyclic forms also followed similar dynamics (Fig. 1B). No obvious differences were observed, implying that any difference in the behaviour between BCN150 and BOS1FL1 could not be attributed to a difference in their growth capability or *in vitro* metacyclogenesis. In addition, to extend the analysis to the differential susceptibility, if any, to the inhibitory effect of ROS depending on the isolate, the parasites were exposed to exogenous

hydrogen peroxide (H_2O_2) stress. The results showed a reduction in the viability (%) of promastigotes in both isolates in a dose-dependent manner (Fig. 2); however, the viability of BCN150 was slightly lower than that of BOS1FL1, a difference that was statistically significant at 4 mM ($P \leq 0.01$). Thus, BOS1FL1 was slightly less susceptible than BCN150 to the effect of ROS *in vitro*, which suggests the potential for a specific selective advantage when faced with host-derived oxidative radicals.

3.2. Differential interaction of *L. infantum* isolates with canine macrophages and monocytes

In this study, we first proposed to investigate whether there are any differences in the behaviour of BCN150 and BOS1FL1 in their interaction with canMø. We infected canMø with promastigotes of both isolates and followed the progression of infection for 72 h. BCN150 had a significantly greater infection index than BOS1FL1 at 24 h (279 ± 18 *vs* 73 ± 7; $P < 0.0001$) and 72 h (176 ± 17 *vs* 53 ± 3; $P = 0.0002$). These differences corresponded with an increased percentage of infected cells by BCN150 and the greatest intensity of infection over 24 and 72 h (Fig. 3A, B).

A similar trend was observed with canMon, with a significantly higher infection index for BCN150 than for BOS1FL1 at 24 h (200 ± 28 *vs* 86 ± 6; $P = 0.0002$) and 72 h (435 ± 16 *vs* 142 ± 14; $P < 0.0001$). According to these results, BCN150 showed the most effective infection capacity in canine macrophages and monocytes, obtaining both a higher percentage and intensity of infection at the two temporal points (Fig. 3A, B).

3.3. Characterization of the oxidative burst after *L. infantum* infection in canine macrophages and monocytes

To assess whether there was a difference in the induction of NO after infection with *L. infantum*, we measured the nitrite levels in canine monocytes and macrophages. However, we could not detect any production in either non-infected cells or cells infected with any of the *L. infantum* isolates/strains. To investigate whether ROS play any role in parasite killing in canine monocytes, we assessed ROS production after infection with both *L. infantum*. The highest production of ROS was obtained in canMon 24 h after infection with BCN150 (mean ± SEM: 295.2 ±7.2). This production was even higher than the levels resulting from the specific induction of ROS by treatment with PMA. In canMø, there was also an enhanced ROS production in response to infection, although it was not greater than the levels induced by the PMA control (Fig. 4). To further investigate whether ROS production could be related to the different intracellular behaviours displayed by BCN150 and BOS1FL1, we performed infections of canMon and canMø treated with PMA before infection. In canine monocytes after 24 h and 72 h, the percentage of infected cells, as well as the overall burden of intracellular parasites, was significantly reduced in the PMA-treated cells (Fig. 5A, B). According to that result, the infection index was significantly reduced compared to that with non-treated cells (Table 1). Furthermore, the PMA reduction effect was higher in the case of BCN150 (Table 1). In canine macrophages, the percentage of infection was only significantly reduced at 24 h after infection, and the parasite load was not only not reduced but also even increased at 72 h in treated cells infected with BOS1FL1 (Fig. 5A, B).

3.4. Modulation of arginase activity and nitric oxide production in canine monocytes and macrophages

To further investigate whether BOS1FL1 or BCN150 could subvert host cell responses that kill intracellular parasites in different ways, we compared the arginase activity levels

in *ex vivo* infected canMon and canMø in co-culture with autologous lymphocytes at 72 h after infection. Unfortunately, as described above for NO production, it was not possible to detect arginase enzyme activity that exceeded the detection limits of the technique in canMon and canMø infected with BOS1FL1 and BCN150. To assess whether the negative results were due to the sensitivity of the method or because the levels were completely undetectable, we evaluated arginase (*Arg*2) and inducible nitric oxide synthase (iNOS) expression after infection of cells followed by analysis of their mRNA expression by qRT-PCR. We could not find a significant level of iNOS expression. In addition, although we found a low expression level of arginase 2 (*Arg*2), it did not differ between cells infected with any isolate and non-infected cells (data not shown).

3.5. mRNA expression pattern of TLR-2, TLR-4 and cytokines

We investigated whether the differences in isolate-specific intracellular behaviour would also impact cytokine levels. Thus, we examined the expression of pro-inflammatory (IL-1β, IL-6, IL-12p40, IL-18, and TNF-α) and anti-inflammatory (IL-10) cytokines in canine monocytes and macrophages following infection with BCN150 and BOS1FL1. Our results showed upregulation of IL-1β and TNF-α production in the infected monocytes compared to uninfected cells. These expression levels were even higher in the case of BCN150, although the differences were not significantly different from those observed with BOS1FL1 (Fig. 6A, B). The expression levels of the other important pro-inflammatory cytokines associated with *L. infantum* infection analysed, such as IL-18, IL-12p40, and IL-6, were only slightly expressed in canine monocytes after parasite infection, with no significant differences between infected cells and non-infected control cells.

Among anti-inflammatory cytokines, we found upregulation of IL-10 in BCN150-infected monocytes with respect to uninfected and BOS1FL1-infected cells (Fig. 6C). Infected macrophages presented very low expression of every cytokine analysed, with no significant differences between the isolates or compared with the control cells.

TLR expression has been associated with leishmanial infection, and we evaluated *ex vivo* expression of TLR-2 and TLR-4 after canine monocyte and macrophage infection. Our results revealed a significant increase in the expression of TLR-2 and TLR-4 in infected monocytes compared to that in uninfected cells. This upregulation was higher in the case of BCN150 infection than BOS1FL1 infection, although the differences were not statistically significant (Fig. 6D). Finally, there were no significant differences in TLR-2 and TLR-4 expression after infection with any of the isolates compared to those in the control group macrophages (Fig. 6D).

3.6. IL-10, TNF-α, and INF-γ production by lymphocytes in co-culture with *L. infantum*-infected canMon and canMø

As a next step, we investigated the balance in the synthesis of anti-inflammatory and pro-inflammatory cytokines resulting from the co-culture of canMon/canMø after infection with parasites. Infection with BCN150 and BOS1FL1 significantly increased pro-inflammatory cytokine (TNF-α, and especially IFN-ɤ) and anti-inflammatory cytokine (IL-10) expressions to levels even higher than those observed with control LPS treatment (Fig. 7). There were no significant differences in TNF-α production between both isolates. However, in both cell types, BCN150 induced significantly higher IL-10 (264 ± 31 *vs* 165 ± 24 pg/mL; $P = 0.0002$) and lower IFN-ɤ (196 ± 3 *vs* 261 ± 26 pg/mL; $P = 0.0151$) production than did BOS1FL1. Taken together, these results indicate that BCN150

370 enhances the anti-inflammatory/pro-inflammatory cytokine secretion rate, thus
371 suggesting a switch to the susceptibility Th2 profile in those cells infected with BCN150.
372
373 **4. Discussion**
374
375 The establishment and outcome of leishmaniosis is intrinsically associated with the
376 balance between the immune response of the host and the parasite's ability to survive.
377 Numerous mechanisms developed by the pathogen are known to modify its virulence,
378 allowing it to evade both innate and adaptive immune responses, which may be implicated
379 in its adaptation to new hosts and the spread of the disease (Gupta et al., 2013). When *L.*
380 *infantum* infection begins, monocytes are one of the first immune host cells to interact
381 with the parasite, and the way in which this interaction occurs could determine infection
382 control or progression (Viana et al., 2013).
383
384 In this study, we speculated whether a change in the host tropism between isolates of
385 *L. infantum* could be responsible for the special features in terms of virulence, outcome
386 and host scope of leishmaniosis in the outbreak of Madrid (Horrillo et al., 2019; Miro et
387 al., 2017). To assess this hypothesis, we compared the behaviour of one isolate
388 (BOS1FL1) and one strain (BCN150) of *L. infantum* during interactions with monocytes
389 and macrophages of canine origin.
390
391 Phenotypic characterization of promastigotes *in vitro* can be relevant to identifying any
392 effects, of growth kinetics or metacyclogenesis, on infection ability other than host
393 interaction. No obvious differences were observed in the *in vitro* growth dynamics or the
394 development of metacyclic forms of BCN150 and BOS1FL1. Furthermore, when

comparing the percentage and intensity of infection obtained when infecting macrophages and monocytes of canine origin, we observed a clear difference between them. BCN150 had a higher infection index than BOS1FL1. Interestingly, these results were opposite to those previously described for both isolates in macrophages and dendritic cells of murine origin (Dominguez-Bernal et al., 2014).

To understand the reasons behind the distinctive differences in intracellular behaviour between BOS1FL1 and BCN150, we first investigated whether they exerted detrimental effects on leishmanicidal host machinery. Thus, we compared the levels of NO and arginase activity in *ex vivo* infected canMon and canMø at 96 h post-infection. We found undetectable NO and arginase production in BOS1FL1- and BCN150-infected canMon and canMø in the presence of LPS. These results were also obtained when mRNA expression of *arg2* and *iNOS* levels were assessed. In both cases, the expression was negligible in infected cells with either isolate and in non-infected cells, as previously described (Singh et al., 2018). While there are studies demonstrating that *Leishmania* killing in murine macrophages is directly related to the activity of NO (Mauel et al., 1991), this fact does not appear to play a significant role in the defence of human monocytes or macrophages, (Almeida et al., 2017; Gantt et al., 2001) being replaced by the synthesis of reactive oxygen species (ROS) (Novais et al., 2014).

Furthermore, different expression levels of NO and arginase in rodent macrophages determine the resistance (for rats) and susceptibility (for mice) to *Toxoplasma gondii* infection, indicating that those mechanisms could be involved in variation in susceptibility and host tropism (Li et al., 2012). In dogs, the information is much more limited, and the role, if any, of NO production in macrophages is controversial since some

authors reported the production of NO (de Almeida et al., 2017; Petitdidier et al., 2016), while others, in agreement with our results, showed undetectable levels of NO production for canMon and canMø infected with *L. infantum* (Martínez-Rodrigo et al., 2019; Montserrat-Sangrà et al., 2018; Turchetti et al., 2015). Our data demonstrate that NO and arginase do not play a role in controlling parasites *in vitro* in canMon and canMø, neither determining the difference observed between the isolates.

However, high ROS production was induced in canMon and canMø after infection. Although there were no significant differences between BCN150 and BOS1FL1, the induction of ROS production highlights the important defensive role of these compounds in the innate responses of canine cells to leishmaniosis, substantiating the hypothesis that the defensive role of the canine monocyte oxidative burst is dependent on ROS and not on NO production.

The role of ROS is relevant for the destruction of a variety of intracellular pathogens, such as *Leishmania*; therefore, the viability of *Leishmania* in the presence of H_2O_2 has been previously analysed (Cortes et al., 2018). More interestingly, there have been reported differences in ROS susceptibility between natural hybrids of *L. infantum* and *L. major* (Cortes et al., 2018). Here, we described a higher resilience to different H_2O_2 concentrations in BOS150 than in BCN1FL1, suggesting the potential for a specific advantage to resistance to the host oxidative radicals or to those immune cells with higher oxidative radical production. Interestingly, after induction of ROS production with PMA, there was a clear increase in killing activity, with reductions both the percentage of infection and the intensity of infection, although this effect was less intense for BOS1FL1, which is more resistant to exogenous hydrogen peroxide (H_2O_2) (Fig. 2). Whether this

445 difference in resistance could be related to a specific selective advantage against
446 differential host-derived oxidative radicals that led to tropism adaptation and difference
447 virulence behaviour needs to be further studied.
448
449 Given that ROS susceptibility data indicated a BCN150/BOS1FL1-specific impact on the
450 innate immune response orchestrated by canMon and canMø, we assessed whether these
451 differences also affect the adaptive immune response. Thus, we next examined the
452 involvement of relevant pro-inflammatory (IL-1β, IL-6, IL-12, IL-18, and TNF-α) and
453 anti-inflammatory (IL-10) cytokines that could explain the differences in the behaviour
454 of the isolates after infection of canMon or canMø. We observed that BCN150 and
455 BOS1FL1 did not induce detectable expression levels of IL-6, IL-12 and IL-18. Similarly,
456 the absence of IL-12 in supernatants of virulent or avirulent *L. major*-infected human
457 macrophages has been reported (Brodskyn et al., 2000). Moreover, strains of *L. major*
458 with different virulence in BALB/c mice and *L. infantum* with viscerotropic and
459 dermotropic tropism displayed similar cytokine-inducing capacities in human monocytes
460 (Meddeb-Garnaoui et al., 2009). Together with our results, these data suggest that the
461 virulence of *Leishmania* does not influence the inhibition of IL-12 production by canine
462 monocytes or macrophages.
463
464 On the other hand, we detected increased expression of IL-1β and TNF-α in infected
465 monocytes; however, although slightly higher expression was displayed in BCN150-
466 infected cells, it was not significantly different between the two isolates. *Leishmania* are
467 poor inducers of IL-1β alone but do promote IL-1β production when macrophages are
468 also stimulated with LPS (Scott and Novais, 2016). We found increased IL-1β expression
469 levels in monocytes infected with *Leishmania* without stimulation with LPS. IL-1β can

also enhance inflammation by promoting TNF-α expression, as we observed in monocytes infected with both *Leishmania* isolates. Similar to TNF-α, IL-1β can play a dual role, leading to protective or pathogenic effects during *Leishmania* infection (Scott and Novais, 2016). Only a few studies have investigated the role of IL-1β in *Leishmania*–infected patients, and these studies have indicated that IL-1β also contributes to disease in humans (Santos et al., 2018). Unfortunately, no data are described involving IL-1β with the exacerbation of canine leishmaniosis. Whether this upregulation in canMon is related to an increase in the severity associated with inflammasome activation must be deciphered in future studies. Finally, IL-10 expression was significantly higher in BCN150-infected canMon than in BOS1FL1-infected canMon. Although some studies have suggested that this cytokine is not associated with disease susceptibility in dogs (Solano-Gallego et al., 2016), many others (de Almeida et al., 2017{Martínez-Rodrigo, 2019 #3167; Martínez-Rodrigo et al., 2019; Medina-Colorado et al., 2017; Reza et al., 2019) are in agreement with our data, where a reduction in the infection index is associated with a decrease in IL-10 expression in canine monocytes. These results were also obtained in the supernatant of infected monocytes and macrophages subsequently co-cultured with autologous lymphocytes, where higher production of IL-10 along with lower INF-γ compatible with a susceptible infectivity phenotype was shown by BCN150, while BOS1FL1 displayed a more resistant profile characterized by lower IL-10 together with higher INF-γ values.

TLRs expressed on innate immune cells are critical for *Leishmania* recognition, which determines the outcome of the infection (Faria et al., 2012). Although most studies appoint a protective role for TLRs, there is growing evidence that in some cases, TLRs facilitate infection (Faria et al., 2012). However, although a higher infection index of

BCN150 was positively associated with increased expression of TLR-4 compared to that of BOS1FL1, this difference was not statistically significant.

While most previous studies focused on the requirement of TLRs for cytokine synthesis and for the development of adaptive immunity, studies on how those receptors contribute to the control of parasite intracellular growth are still largely absent (Faria et al., 2012). In this study, we could not associate a significant role in the upregulation of TLRs with the different intracellular behaviours of BCN150 and BOS1FL1. Furthermore, several studies have shown a role for TLRs in the generation of ROS during *Leishmania* infection (Srivastava et al., 2012). Therefore, increased ROS production in canine infected cells could be due to increased expression of TLRs in monocytes differentially driven by BOS1FL1 or BCN150; however, we did not find any association between upregulation of TLR-4 and the slight increase in ROS production with BCN150.

The immune response triggered by infection of canine monocytes with one isolate from the human leishmaniosis outbreak did not differ significantly from the traditional response described in canine target cells by traditional isolates (Gradoni, 2015; Reis et al., 2010). The results obtained from the cytokine, TLR, and oxidative burst profiles do not seem to account for the reduced infection index observed in BOS1FL1 infected cells and therefore do not explain the changes observed in terms of epidemiology, pathology and host-tropism preference described in the outbreak in Madrid. The virulence factors involved in the differential outcome of infection with different *L. infantum* strains are still poorly defined.

5. Conclusions

These findings provide insights to improve the understanding of the mechanisms used by *L. infantum* to subvert canine mammalian cell defence tools. Furthermore, the differences in cytokine profiles and ROS susceptibility between isolates indicate that these mechanisms may also account for variation in susceptibility of individuals within the natural population of *L. infantum*. Nevertheless, the different behaviour described for *L. infantum* isolates according to the mammal origin of the host cells must be related to factors beyond the different expression levels of iNOS, Arg2 and TLR profiles. Therefore, further studies analysing new virulence factors and pathogenic mechanisms for host and tropism adaptation are necessary to elucidate how isolates modify their virulence and finetune their adaptation to new ecological niches, vectors and host and tissue tropism.

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Disclosure statement

The authors declare that they have no competing interests.

Author contributions

Conceptualization, AM, GDB and JC; methodology, AM and AMR; formal analysis, AM and AMR; resources, LMV and JAO; writing—original draft preparation, AM and GDB;

writing—review and editing, AMR and JAO; supervision, GDB and JC.; funding acquisition, GDB and JC.

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735

Table 1. Effect of PMA treatment on the infection index in canine monocytes and macrophages.

Infection index	canMon				canMø			
	BCN150		BOS1FL1		BCN150		BOS1FL1	
	Untreated cells	PMA	Untreated cells	PMA	Untreated cells	PMA	Untreated cells	PMA
24h after infection	286 ± 15	18 ± 2**	152 ± 13	26 ± 1**	449 ± 3	328 ± 10	332 ± 11	288 ± 17
72h after infection	298 ± 34	42 ± 4**	161 ± 12	37 ± 9**	414 ± 4	432 ± 38	330 ± 20	500 ± 30**

CanMon and CanMø were pre-treated with PMA at 1 µg/mL over 30 min and infected with *L. infantum* BCN150 or BOS1FL1 at a ratio of 5:1 parasites:cells. Statistical analysis was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. Asterisks (*) indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).

Supplementary Table 1 (S1). List of sequences of primers used for cytokine real-time PCR (qPCR) and standard curve data.

Target	Primer	Sequence (5'→ 3')	Orientation	Position	Product (bp)	NCBI[a] Accession number	Reference
β-actin	qmB-actin-1D	GGCACCACACCTTCTACAATG	Forward	343-363	151	NM_001195845.2	This work
	qmB-actin-1R	TGGATGGCTACGTACATGGCT	Reverse	476-496			
GAPDH	qGAPDH-1D	CCAGAACATCATCCCTGC	Forward	603-620	240	AB038240.1	This work
	qGAPDH-1R	GAGACCACCTGGTCCTCAGTG	Reverse	822-842			
IL-1β	qIL-1 β-1D	GATGGCTTACTCCAGTAACAATG	Forward	1429-1451	158	XM_005630074.2	This work
	qIL-1 β-1R	GCCTCAGACTCTTGTTACAGAGC	Reverse	1564-1586			
IL-6	qIL-6-1D	GAGACCTGCTTGACAAGAATCAC	Forward	367-389	123	NM_001003301.1	This work
	qIL-6-1R	GATCTTGGTACTCATGTGCACAG	Reverse	467-489			
IL-10	qIL-10-1D	CACGACCCAGACATCAAGAACCAC	Forward	304-327	142	EU426968.1	This work
	qIL-10-1R	TACTAAATGCGCTCTTCACCTGC	Reverse	423-445			
IL-12	qIL-12-1D	AGCAGTGACACTTTCAGCAGAG	Forward	513-534	143	NM_001003292.1	This work
	qIL-12-1R	GCTTGTGAATAGCATCCACCACG	Reverse	633-655			
IL-18	qIL-18-1D	GATTCTGACTGTACAGATAATGC	Forward	211-233	99	NM_001003169.1	This work
	qIL-18-1R	CTTCACAGAGATAGTTACTGCCAG	Reverse	286-309			
TNF-α	qTNFa-1D	AGCCAGTAGCTCATGTTGTAGC	Forward	260-281	122	NM_001003244.4	This work
	qTNFa-1R	CGGCACTATCAGCTGGTTGTCTG	*Reverse*	*359-381*			
Arginase	qArg-1D	GCATGGCTATTGGAAGCATCTC	Forward	794-815	182	XM_532053.6	This work
	qArg-1R	GAATCCTGGTACATCGGGTATC	Reverse	954-975			
iNOS	qInos-1D	GTCTTGTCTGGGAGCCATCATG	Forward	586-607	161	XM_022422531.1	This work
	qInos-1R	CCACCCTGGCCAGATGTTCCTC	Reverse	725-746			
TLR2	qTLR 2-1D	GAACTTATCCGACACAAGAATGC	Forward	1331-1353	164	EU487534.1	This work
	qTLR 2-1R	AGGCATCTGGTAGAGTCTTCAAC	Reverse	1472-1494			
TLR4	qTLR 4-1D	ATTCCAGTTTGAAGCAGGCCAGTG	Forward	1300-1323	134	NM_001002950.2	This work
	qTLR 4-1R	GACTTCGAGGCTGACCAAGCCATC	Reverse	1410-1433			

[a]NCBI accession numbers are for cDNA sequences used in primer design (https://www.ncbi.nlm.nih.gov/nuccore).

752 **Figure captions**

753

754 **Figure 1. Growth dynamics (A) and *in vitro* metacyclogenesis development (B) of the**
755 ***Leishmania infantum* isolates BCN150 and BOS1FL1**. Promastigotes of each isolate
756 were counted using a Neubauer chamber and a 1/100 dilution. Parasites were considered
757 metacyclic when presenting a flagellum/cell body length ratio >2. The results are
758 represented by the mean values and standard errors of the mean of two replicates from
759 three independent assays. Statistical analysis was performed with Student's *t* test.
760 Asterisks (*) indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).

761

762 **Figure 2. Parasite viability (%) of the BCN150 and BOS1FL1 isolates exposed to**
763 **H_2O_2**. Promastigotes of the *L. infantum* isolates BCN150 and BOS1FL1 were exposed to
764 an increasing concentration of H_2O_2. The viability percentage shown was obtained from
765 one representative of four independent experiments with four replicates each. Statistical
766 analysis was performed with Student's *t* test. Asterisks (*) indicate statistically significant
767 differences (*$P < 0.05$, **$P < 0.01$).

768

769 **Figure 3. Percentage of infection and intensity of infection in canine monocytes and**
770 **macrophages.** canMon and canMø were infected with *L. infantum* BCN150 or BOS1FL1
771 at a ratio of 5:1 parasites:cells. Data from one representative of four independent
772 experiments are shown. The percentage of infected cells **(A)** and the mean number of
773 amastigotes per infected cell **(B)** were evaluated. Statistical analysis was performed with
774 one-way ANOVA followed by Tukey's test for multiple comparisons. Asterisks (*)
775 indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).

776

Figure 4. ROS generation by canine monocytes and macrophages after infection with *L. infantum*. canMon **(A)** and canMø **(B)** were infected with *L. infantum* BCN150 or BOS1FL1 at a ratio of 5:1 parasites:cells. After 24 h or 72 h of infection, cells were treated with DHE (10 µM) for 30 minutes. PMA (1 µg/mL) was used as a positive control. The results are represented by the mean fluorescence intensity (MFI) of two replicates from one assay representative of three independent experiments collected using flow cytometry and analysed using FlowJo software. Statistical analysis was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. Asterisks (*) indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).

Figure 5. Effect of PMA treatment on the percentage of infection and intensity of infection in canine monocytes and macrophages. canMon and canMø were pre-treated with PMA at 1 µg/mL over 30 min and infected with *L. infantum* BCN150 or BOS1FL1 at a ratio of 5:1 parasites:cells. Data from one representative of four independent experiments are shown. The percentage of infected cells **(A)** and the mean number of amastigotes per infected cell **(B)** were evaluated. Statistical analysis was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. Asterisks (*) indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$) between the untreated and PMA-treated groups.

Figure 6. mRNA expression of anti-inflammatory (IL-10) or proinflammatory (IL-1β, TNF-α) cytokines and TLR-2 and TLR-4 in canine monocytes and macrophages. Graphs represent the relative quantification by the comparative $2^{-\Delta\Delta Ct}$ method of IL-1β **(A)**, TNF-α **(B)**, IL-10 **(C)**, and TLR-2 and TLR-4 **(D)** mRNA expression levels (fold change in expression) in canMon and canMø challenged for 72 h with *L. infantum*

802 BCN150 or BOS1FL1 (at a ratio of 5:1) in relation to those in non-infected cells. The
803 results show the mean of four independent experiments. Statistical analysis was
804 performed with one-way ANOVA followed by Tukey's test for multiple comparisons.
805 Asterisks (*) indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).
806
807 **Figure 7. IL-10, TNF-α, and INF-γ production by lymphocytes in co-culture with**
808 ***L. infantum*-infected canine monocytes and macrophages.** The graph represents the
809 concentration of cytokines in supernatants obtained from lymphocytes in co-culture with
810 non-infected cells or canine monocytes **(A)** or macrophages **(B)** challenged with
811 *L. infantum* BCN150 or BOS1FL1. Each column and error bar represent the mean and
812 SEM of three replicates from three independent experiments. Statistical analysis was
813 performed with two-way ANOVA followed by Tukey's test for multiple comparisons.
814 Asterisks (*) indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).