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Expression of key cytokines in dog macrophages infected by *Leishmania tarentolae* opening new avenues for the protection against *Leishmania infantum*

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The detection of *Leishmania tarentolae* in sympatric areas where *Leishmania infantum* is endemic raised questions regarding the protective effect exerted in dogs by *L. tarentolae* when in coinfection. This study aimed monitoring the in vitro gene expression of pro- (IFN- γ; TNF-α; IL-12) and anti-inflammatory (IL-4; IL-6; IL-10) cytokines in primary canine macrophages infected by *L. tarentolae* and *L. infantum* in single and in co-infections. Macrophages differentiated from dog blood mononuclear cells were infected with the *L. tarentolae* field-isolated (RI-325) and laboratory (LEM-124) strains, with *L. infantum* laboratory strain (IPT1), or both. Infection and the number of amastigotes per infected cell were evaluated microscopically by counting a total of 200 cells between 4 and 96 h. Cytokine gene expression was analyzed by real-time PCR from infected macrophages mRNA. Single infections presented higher expression of the cytokines IL-4 and IL-6, and lower of IL-12. Co-infections induced a lower gene expression of IL-4 and IL-6, and a higher gene expression of IL-12, correlating with the low amastigote burden despite the slight increase of infected cells. Data highlight the potential protective effect of *L. tarentolae* against *L. infantum* in co-infection by the reduced anti-inflammatory and increased pro-inflammatory cytokines gene expression, opening new perspectives for a canine vaccine development exploiting the non-pathogenic *L. tarentolae*.

Leishmaniases are neglected, vector-borne tropical diseases caused by *Leishmania* spp. (class of Kinetoplastida, family of Trypanosomatidae), with more than 350 million people at risk of infection worldwide[1]. The genus *Leishmania* includes more than 20 species capable to infect and replicate in dendritic cells and macrophages of vertebrate hosts[2–6], causing different clinical manifestations. *Leishmania infantum*, the main etiological agent of visceral leishmaniasis in humans, is the most widespread species of zoonotic concern that finds its reservoir in dogs, as well as in other mammals[7,8]. This disease is prevalent in poor socio-economic contexts (e.g. East Africa, Southeast Asia, and Latin America), and in several Mediterranean countries[9,10], with a distribution overlapping with that of its sand fly vectors, *Phlebotomus* spp. and *Lutzomyia* spp. in the Old and New Worlds, respectively[11,12]. In dogs, canine leishmaniasis (CanL) caused by *L. infantum,* is widespread also in high-income countries, in tropical and temperate areas. Other *Leishmania* species, such as those found in reptiles (subgenus *Sauroleishmania*), may also occur in the same geographical areas of *L. infantum*[13]. For example, *Leishmania tarentolae* has been firstly described in *Tarentola mauritanica* gecko[14] and was later detected in other reptiles, including *Podarcis filfolensis* and *Podarcis siculus* lizards[15–17]. As indicated by molecular and serological evidence, other hosts such as dogs and humans are exposed to this non-pathogenic species, as indicated by its detection using molecular and serological methods[17,18]. This suggests that this species may adapt to different vertebrate organisms. *Leishmania tarentolae* is vectored by *Sergentomyia minuta*[17], a sand fly that feeds preferentially on reptiles and is highly prevalent in Mediterranean countries[19]. Nonetheless, *S. minuta* has also been reported to feed on mammals[19,20], which may account for the detection of *L. tarentolae* DNA in human and dog blood[13,18,21,22].

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In vitro studies showed that *L. tarentolae* is capable of infecting murine and human macrophages and dendritic cells[2–5]. Recently, this species has also been shown to infect and persist in primary cultures of canine macrophages[23]. The latter finding, coupled with the detection of *L. tarentolae* in sympatric areas where *L. infantum* is endemic, raised questions regarding the potential protective effect that the non-pathogenic species may exert towards CanL in dogs co-infected with the pathogenic *L. infantum*[6,13]. The outcomes of such mixed infections may depend on various factors, including the balance between the host Th1 and Th2 immune responses[24–27]. Indeed, macrophages can be polarized into activated M1 or alternatively activated M2 phenotypes depending on cytokines stimuli[28]. In particular, a Th1 subset mediated by IFN-γ, TNF-α and IL-12 is suggested to promote the macrophage activation against parasite infection, limiting the infection and therefore the severity of clinical presentation[29]. On the other hand, the Th2 immune response, characterized by the production of cytokines such as IL-4 and IL-10, shift macrophages towards the M2 phenotype, suppressing the M1 activation and allowing the parasite to survive and multiply within these cells, possibly causing severe clinical manifestations[30]. Although IL-6 is often associated with pro-inflammatory response, this cytokine may also contribute to anti-inflammatory processes by modulating macrophage polarization. Indeed, IL-6 may shift toward either M1 or M2 macrophages phenotype, depending on the presence of other immune cells or cytokines signaling[31].

Here, we exploit our previously established experimental infection protocol using primary canine macrophages[23] to compare the cytokine profiles in response to infection with either *L. infantum*, or *L. tarentolae*, or both. We monitored the gene expression of the pro-inflammatory cytokines IFN-gamma (IFN-γ), TNF-alpha (TNF-α) and IL-12[32,33] that are associated with a protective Th1 response, as well as IL-4, IL-10 and IL-6 whose gene expression is generally linked to clinical disease[34].

Results

Infection and parasite burden parameters

Changes in the percentage of infected dog macrophages and the number of intracellular parasites per infected cell (a/infc) were evaluated 96 h after the infection with *L. infantum* and/or *L. tarentolae*. For each *Leishmania* strain, the percentage of infected macrophages and a/infc is shown in Fig. 1.

Specifically, among *L. tarentolae* strains, RI-325 presented a significatively higher number of infected macrophages in all time points when compared with LEM-124 (Fig. 1A), except at 96 h where the infection rate is the same for both strains. From 8 h time point, IPT1 showed a significantly ($P<0.0001$) higher number of infected macrophages when compared with the other lab strain LEM-124. From 24 h, coinfections with RI-325 + IPT1 showed a significant ($P<0.01$) higher percentage of cell infection in comparison with LEM-124 + IPT1. The medium value of a/infc was higher for IPT1 (1.549 a/infc) in comparison with *L. tarentolae* strains in single (up to 1.363 a/infc, $P<0.001$) and in coinfection conditions (up to 1.364 a/infc, $P<0.0001$). Significant differences in a/infc were observed mainly between LEM-124 in single and in coinfection with IPT1 ($P<0.001$), and between IPT1 in coinfection with RI-325 ($P<0.0001$) (Fig. 1B).

mRNA expression of anti- and pro-inflammatory cytokines

A lower relative gene expression of the anti-inflammatory cytokine IL-4 was observed for both *L. tarentolae* (i.e., up to 6.681 relative units for RI-325 and 3.041 for LEM-124 at 96 h) in comparison with IPT1 single infection (8.481 relative units at 24 h), along with a decrease in coinfections for both *L. tarentolae* strains until 96 h post-infection (i.e., 0.003 relative units for RI-325 + IPT1; 0.217 relative units for LEM-124 + IPT1, $P<0.0001$) (Fig. 2A and B; Table 1). A reduction of IL-6 gene expression was detected in coinfections for all *Leishmania* strains (Fig. 2C and D; Table 1), from which the gene expression was significantly lower between coinfections (0.172 for RI-325 + IPT1, $P<0.001$ at 96 h) and single infections (11.622 relative units for IPT1, $P<0.0001$ at 96 h). In addition, the gene expression of IL-6 was statistically higher at 4 h (26.611 relative units, $P<0.0001$)

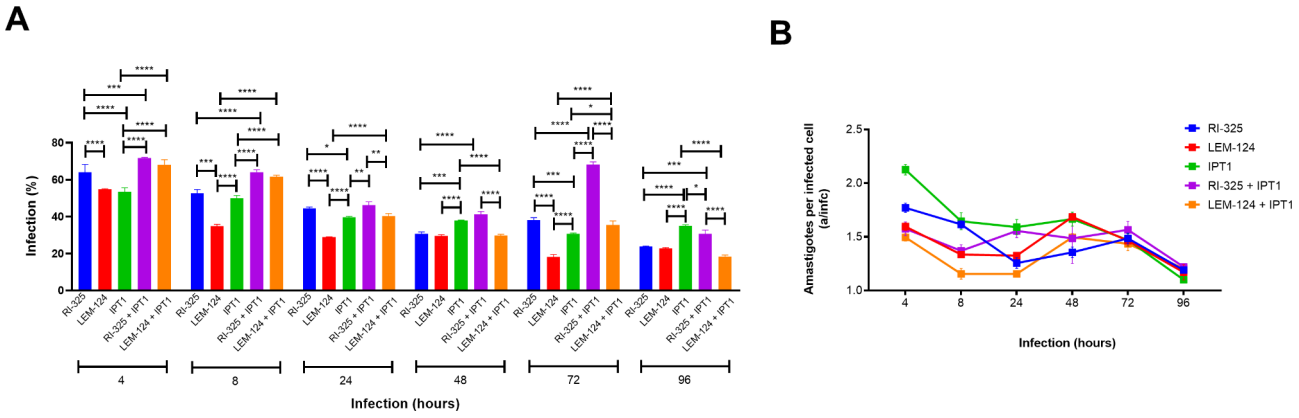

Fig. 1. *Leishmania* infection efficiency (**A**) and intracellular parasite burden at 96 h post-infection (**B**) were assessed microscopically in primary dog macrophages performed in triplicate. RI-325 and LEM-124: *Leishmania tarentolae*; IPT1: *Leishmania infantum*. a/infc: average number of amastigotes in each infected cell. Asterisks indicate the statistical difference between groups (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$).

on RI-325 than both laboratory strains (17.644 relative units for LEM-124; 18.332 relative units for IPT1 at 4 h). The highest gene expression value for IL-10 was noticed for all *Leishmania* strains at 4 h in single and coinfected conditions, with a constant decrease over the assessed time points (Fig. 2E and F; Table 1). The highest gene expression values of the pro-inflammatory IL-12 was detected for dog macrophages infected with the LEM-124 strain (7.096 relative units) at 4 h and on RI-325 + IPT1 coinfection at 4 h (5.905 relative units) and 96 h (6.705 relative units) (Fig. 3; Table 2). Overall, the IL-12 gene expression values in coinfections conditions were significantly higher ($P < 0.0001$; up to 6.705 relative units for RI-325 + IPT1 at 96 h; up to 4.699 relative units for LEM 124 + IPT1 at 4 h) than those observed in single infection with IPT1 (2.261 relative units). The pro-inflammatory cytokines IFN-γ and TNF-α were not detected.

Discussion

The results herein reported suggest that *L. tarentolae* may elicit the production of a cytokine profile that could ultimately exert a protective effect against *L. infantum* infection. This is highlighted by the reduced expression of

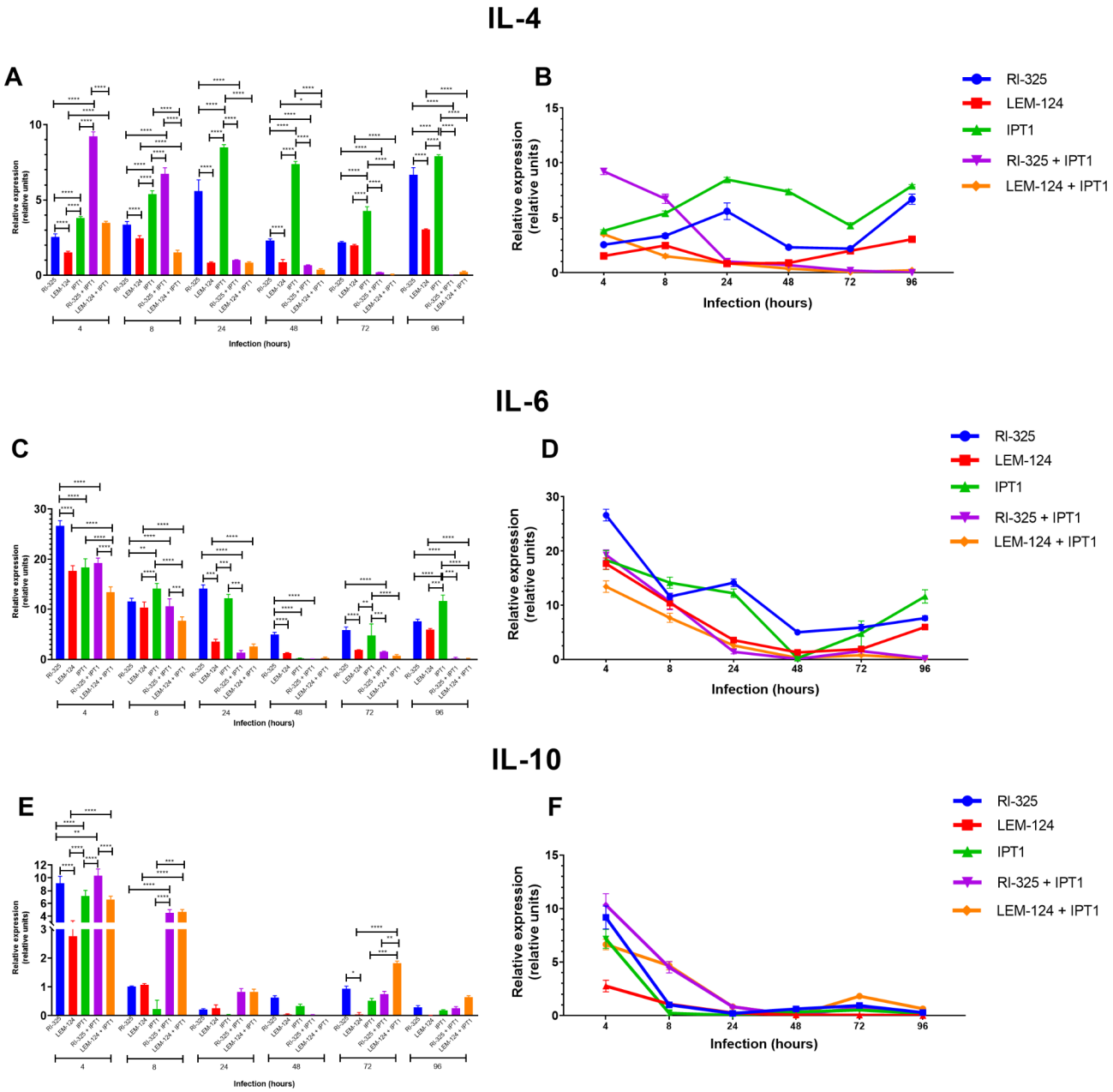

Fig. 2. Relative mRNA gene expression (relative units) of anti-inflammatory cytokines IL-4 (**A**, **B**), IL-6 (**C**, **D**) and IL-10 (**E**, **F**) for each strain and its coinfections from 4 to 96 h in triplicate (standard deviation shown). RI-325 and LEM-124: *Leishmania tarentolae*; IPT1: *Leishmania infantum*. Asterisks indicate the statistical difference between species/strains (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

	IL-4														
	Leishmania tarentolae						*Leishmania infantum*			*L. tarentolae* + *L. infantum*					
	RI-325			LEM-124			IPT1			RI-325 + IPT1			LEM-124 + IPT1		
Infection (hours)	min	med	max	min	med	max	min	med	max	min	med	max	min	med	max
4	2.305	2.540^{a}	2.706	1.477	1.519^{b}	1.605	3.502	3.804^{c}	4.023	8.985	9.224^{d}	9.562	3.400	3.493^{c}	3.590
8	3.200	3.354^{a}	3.605	2.350	2.468^{b}	2.653	5.170	5.395^{c}	5.610	6.540	6.720^{d}	7.201	1.331	1.505^{e}	1.652
24	4.750	5.589^{a}	6.205	0.784	0.830^{b}	0.905	8.342	8.481^{c}	8.702	0.998	1.017^{b}	1.032	0.799	0.848^{b}	0.905
48	2.210	2.310^{a}	2.420	0.705	0.882^{b}	1.038	7.305	7.365^{c}	7.590	0.650	0.677^{bd}	0.705	0.303	0.368^{d}	0.450
72	2.125	2.183^{a}	2.256	1.908	1.988^{a}	2.036	3.985	4.281^{b}	4.532	0.173	0.193^{c}	0.210	0.313	0.050^{c}	0.085
96	6.302	6.681^{a}	7.202	2.997	3.041^{b}	3.095	7.815	7.905^{c}	8.105	0.001	0.003^{d}	0.006	0.174	0.217^{d}	0.292
	IL-6														
	Leishmania tarentolae						*Leishmania infantum*			*L. tarentolae* + *L. infantum*					
	RI-325			LEM-124			IPT1			RI-325 + IPT1			LEM-124 + IPT1		
Infection (hours)	min	med	max	min	med	max	min	med	max	min	med	max	min	med	max
4	25.423	26.611^{a}	27.506	16.520	17.664^{b}	18.570	16.428	18.332^{bc}	19.878	18.228	19.273^{bd}	20.070	12.520	13.424^{e}	14.592
8	11.205	11.588^{a}	12.308	9.250	10.383^{a}	11.360	13.025	14.150^{b}	15.026	9.418	10.641^{a}	9.418	6.912	7.665^{c}	8.563
24	13.580	14.114^{a}	14.952	3.074	3.558^{b}	4.021	11.472	12.193^{a}	13.025	1.250	1.410^{c}	1.850	1.985	2.581^{bc}	2.908
48	5.134	5.011^{a}	5.320	1.210	1.296^{b}	1.376	0.149	0.231^{b}	0.302	0.045	0.075^{b}	0.120	0.063	0.285^{b}	0.440
72	5.205	5.883^{a}	6.235	1.890	1.915^{b}	1.950	2.770	4.772^{a}	7.311	1.410	1.546^{b}	1.647	0.560	0.767^{b}	0.985
96	7.205	7.625^{a}	8.012	5.808	5.972^{a}	6.204	10.629	11.622^{b}	12.985	0.026	0.192^{c}	0.465	0.123	0.172^{c}	0.252
	IL-10														
	Leishmania tarentolae						*Leishmania infantum*			*L. tarentolae* + *L. infantum*					
	RI-325			LEM-124			IPT1			RI-325 + IPT1			LEM-124 + IPT1		
Infection (hours)	min	med	max	min	med	max	min	med	max	min	med	max	min	med	max
4	8.125	9.174^{a}	10.260	2.140	2.766^{b}	3.142	6.251	7.173^{c}	8.011	9.205	10.345^{c}	11.250	6.091	6.651^{d}	7.020
8	0.980	1.006^{a}	1.036	1.036	1.069^{a}	1.125	0.049	0.233^{a}	0.586	4.175	4.515^{b}	5.120	4.264	4.676^{b}	5.012
24	0.188	0.211^{a}	0.250	0.126	0.256^{a}	0.365	0.021	0.032^{a}	0.042	0.710	0.833^{a}	0.914	0.755	0.835^{a}	0.930
48	0.536	0.620^{a}	0.680	0.036	0.049^{a}	0.069	0.293	0.336^{a}	0.410	0.014	0.022^{a}	0.041	0.008	0.009^{a}	0.010
72	0.845	0.937^{a}	1.021	0.009	0.047^{b}	0.121	0.453	0.525^{ab}	0.601	0.645	0.746^{ab}	0.841	1.758	1.823^{c}	1.906
96	0.241	0.289^{a}	0.360	0.010	0.008^{a}	0.011	0.174	0.189^{a}	0.210	0.267	0.259^{a}	0.312	0.602	0.644^{a}	0.702

Table 1. Relative gene mRNA expression (relative units) of anti-inflammatory cytokines normalized according to *G3PDH* housekeeping values. Statistically differences ($P < 0.05$) between strains on each time point are indicated. Different letters indicate significative differences. Same letters indicate no significative difference. min: minimal value of gene expression; med: medium value of gene expression; max: maximum value of gene expression.

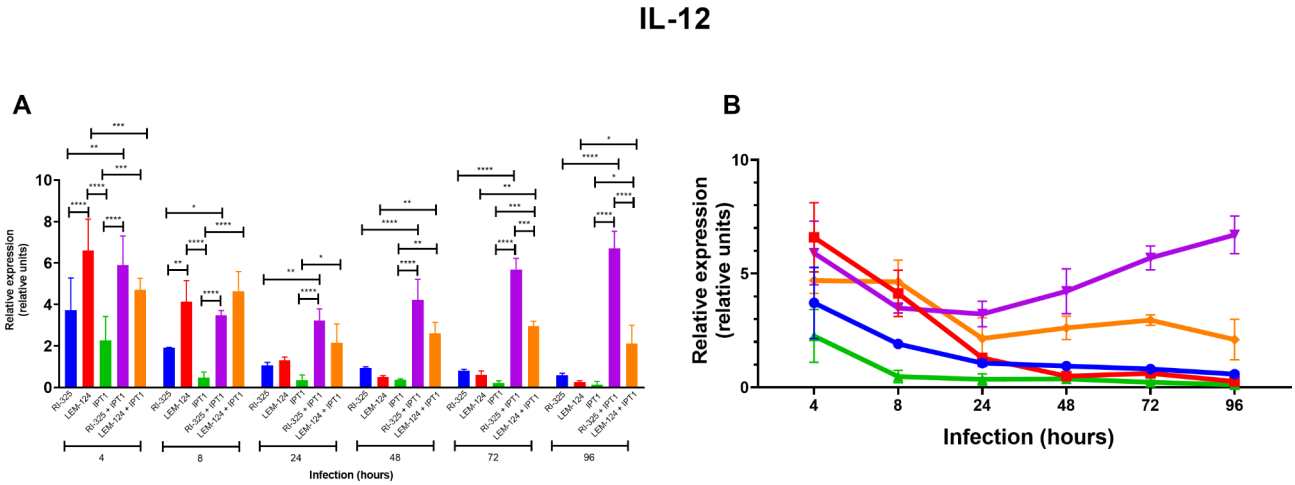

Fig. 3. Relative mRNA gene expression (relative units) of pro-inflammatory cytokines IL-12 (**A**, **B**) for each strain and its coinfections from 4 to 96 h in triplicate (standard deviation shown). RI-325 and LEM-124: *Leishmania tarentolae*; IPT1: *Leishmania infantum*. Asterisks indicate the statistical difference between groups (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$).

anti-inflammatory cytokines (i.e., IL-4 and IL-6) coupled with an increased expression of the pro-inflammatory cytokine IL-12 after 96 h of coinfection in primary canine monocyte-derived macrophages.

The lowest expression of both IL-4 and IL-6 anti-inflammatory cytokines in coinfections (RI-325 + IPT1), in relation to single infection by IPT1 at 24 h, is suggestive of a protective role of *L. tarentolae*. Accordingly, the higher gene expression of pro-inflammatory IL-12 observed in coinfections than in single IPT1 infection throughout all time points may suggest that *L. tarentolae* effectively contributes to an increase in pro-inflammatory response probably due to a sum effect between both species, further increasing the percentage of infected cells. This possibility is further supported by the lower mean value of a/infc observed in coinfection than in single IPT1 infection (with both RI-325 + IPT1 and LEM-124 + IPT1, $P<0.0001$). Indeed, although a strong competitiveness between these two *Leishmania* spp. in infected cells has been previously suggested[35,36], a similar infection efficiency of *L. infantum* and *L. tarentolae* was inferred by the percentual of infection mainly at 72 h. This data also highlights the potential of the RI-325 to induce an anti-inflammatory response by IL-4 and IL-6 cytokines gene expression. In addition, the general higher infectivity of RI-325 compared to *L. infantum* in single infections (i.e., at 4, 8, 24 and 72 h) confirms previous studies[23], ultimately explaining the difference in higher infection observed in coinfected cells (up to 69.2% for RI-325 + IPT1 at 72 h) than in *L. infantum* single infection, even if the number of a/infc is higher on IPT1 than RI-325. This increase of the number of infected cells at 72 h was previously reported[23], which could be mainly by a persistent uptake of amastigotes released in the medium by the macrophage cells despite a decrease in the number of infected cells. Furthermore, the overall decrease in IL-10 gene expression after 8 h post infection among all *Leishmania* strains is not surprising, considering that the role of this cytokine in the disease progression is controversial[24,27,37], at least in in vitro models.

The absence of the gene expression of pro- inflammatory IFN-γ and TNF-α, which are considered key cytokines in the host's protective immune response against *Leishmania* infection[38], could be due to the inhibitory effect mediated by IL-4, IL-6 and IL-10[25,27,29,39,40]. Furthermore, the absence of gene expression of IFN-γ is expected, once the macrophages are not the main producers of this cytokine, and its transcription may require more than 96 h[41]. Conversely, even if the protective effect may be partially due to the TNF-α, this cytokine could not be detected probably due to the rapid turnover and quick degradation of its mRNA, being not always considered as a reliable marker in in vitro model[27,42]. Indeed, the growth factor-free process of macrophage differentiation did not seem to influence the cytokine gene expression herein reported, as highlighted also in previous studies[25].

In the in vitro study design herein conducted, the potential protective effect of *L. tarentolae,* when in coinfection with *L. infantum,* is suggested by the decrease of IL-4 and IL-6 and the increase of IL-12 in coinfections. The partial protection against infection when two species of *Leishmania* coinfect the same individual host was already demonstrated, where a primary infection with *Leishmania tropica* induces partial protection against *Leishmania major* when coinfecting mice[43]. Furthermore, cross-reacting immune responses and the potential protection between taxonomically different *Leishmania* species was also previously observed in an experimental study with Rhesus monkeys, where *L. major* induces significant protection for *Leishmania amazonensis* and *Leishmania guyanensis*[44].

The in vitro mechanisms underlying the low anti-inflammatory and a high pro-inflammatory response to *L. tarentolae* when in coinfection with *L. infantum* needs further investigations and may explain hindrances in interpreting cytokine gene expression profile in canine leishmaniasis under natural conditions[45], where mixed Th1/Th2 responses induced by *L. infantum* have been reported in both asymptomatic and symptomatic dogs, with high mRNA levels observed for IL-4, IL-10 and TNF-alpha in dogs with clinical manifestation of CanL. Ultimately, these results may explain the recorded cases of asymptomatic dogs coinfected with *L. tarentolae* and

	IL-12														
	Leishmania tarentolae						*Leishmania infantum*			*L. tarentolae* + *L. infantum*					
	RI-325			LEM-124			IPT1			RI-325 + IPT1			LEM-124 + IPT1		
Infection (hours)	min	med	max	min	med	max	min	med	max	min	med	max	min	med	max
4	2.445	3.714^{ab}	5.464	4.377	7.096^{c}	9.781	1.080	2.261^{a}	3.402	4.816	5.905^{cd}	7.708	4.102	4.699^{bd}	5.169
8	1.922	1.908^{ab}	1.923	3.092	4.132^{c}	5.123	0.200	0.474^{a}	0.737	3.325	3.490^{bc}	3.796	3.239	4.643^{c}	5.230
24	0.938	1.066^{ab}	1.210	1.162	1.297^{ab}	1.490	0.165	0.352^{b}	0.495	2.784	3.228^{c}	4.023	1.021	2.146^{ac}	3.259
48	0.907	0.939^{abc}	1.002	0.425	0.495^{ab}	0.567	0.394	0.373^{b}	0.401	3.232	4.225^{d}	5.212	2.321	2.619^{cd}	3.212
72	0.792	0.809^{a}	0.875	0.423	0.616^{a}	0.782	0.123	0.223^{a}	0.312	5.092	5.694^{b}	6.063	2.734	2.959^{c}	3.214
96	0.482	0.580^{ab}	0.685	0.223	0.262^{ab}	0.324	0.002	0.115^{b}	0.320	6.032	6.704^{c}	7.623	1.240	2.104^{a}	3.023

Table 2. Relative gene mRNA expression (relative units) of pro-inflammatory cytokines normalized according to *G3PDH* housekeeping values. Statistically differences ($P < 0.05$) between strains on each time point are indicated. Different letters indicate significative differences. Same letters indicate no significative difference. min: minimum value of gene expression; med: medium value of gene expression; max: maximum value of gene expression.

L. infantum[13], suggesting the possibility of genetic exchange and hybridization in sympatric areas, and opening new perspectives for vaccine development against CanL and other leishmaniases.

Materials and methods

Parasites and cultures

Leishmania tarentolae strain isolated from *T. mauritanica* gecko (RTAR/IT/21/RI-325) in Italy, and the laboratory strains of *L. tarentolae* (RTAR/IT/81/ISS21-G.6c/LEM-124) and *L. infantum* zymodeme MON-1 (MHOM/TN/80/IPT1) at 6th passage were cultivated in 1 mL of blood agar and Tobie-Evans, supplemented with Fluorocytosine (250 µg/mL), Gentamicin (250 µg/mL), and Fetal Bovine Serum (FBS, 5%). The cultures were maintained at 26 °C for 5 days before cell infection.

Dog blood sample and clinical examination

Given the aims of the study, in order to avoid further interference caused by biological factors (e.g., age, nutrition, gender) only one dog was used as blood source. The dog was seven-year-old male German Shepherd, after a written informed consent from the owner (one of the authors of the study, D.O.) and he was included being assessed as negative for *Leishmania* spp. infection by indirect immunofluorescent antibody test (IFAT) and by duplex real-time PCR (dqPCR)[18,46]. The study protocol was approved by the ethical committee of the Department of Veterinary Medicine of the University of Bari, Italy (Prot. Uniba 21/2022). The methods to handle animal cells were carried out in accordance with the ARRIVE guidelines and with international, national, and/or institutional regulations.

Generation of macrophages from canine peripheral blood monocytes

Mononuclear cells were obtained according to procedures previously described[23]. Briefly, a total of 10 mL of canine blood was collected in Ethylene Diamine Tetra Acetic acid (EDTA) tubes (BD Vacutainer, San Diego CA, USA) and mixed in equal proportions with RPMI-1640 medium (Euroclone, Milan, Italy). The mixture was subsequently centrifugated with double the volume of Lympholyte®-H (Cedarlane, Italy) at 400 × g for 45 min at room temperature (RT). The resulting buffy coat layer was subjected to two successive washes using 15 mL of warm RPMI-1640 medium, with centrifugation steps performed at 310 × g and 210 × g for 10 min at RT, respectively. The obtained peripheral blood mononuclear cells (PBMC) were suspended in warm RPMI-1640 medium supplemented with FBS (10%) and a mix of antibiotics (100× of Penicillin-Streptomycin). Cells were seeded at a concentration of 2×10^6 cells/mL into 24-well plates containing sterile coverslips. Approximately 10% of the cells differentiated into monocyte-macrophages during the incubation period, and were confirmed by the morphology (e.g., larger and irregular size), cytoplasmic features (i.e., amount of cytoplasm, phagocytic activity) and nuclear form (e.g., larger and irregularly shaped)[47]. Furthermore, cells were also plated in wells without coverslips for subsequent scraped cells analyses. The plates were incubated at 37 °C with 5% CO_2 for 24 h (h) for the adhesion of the mononuclear cells. Subsequently, the medium was replaced to remove non-adherent cells, and 1 mL of fresh RPMI-1640 supplemented with 10% of FBS and 10% of antibiotics mix was added. The plate was incubated for 120 h at 37 °C with 5% CO_2 for complete macrophage maturation. No stimulation factor was used as the cells exhibit adequate differentiation in culture for this short period.

Infection of primary canine monocyte-derived macrophages by *Leishmania* spp.

Leishmania tarentolae (LEM-124 and RI-325 strains) and *L. infantum* (IPT1 strain) promastigotes at the stationary phase of growth were centrifuged at 3000 × *g* for 10 min at RT, followed by three washes with sterile PBS (1×). The resulting pellet was resuspended in RPMI-1640 and counted in a Burker chamber at a dilution ratio of 1:100. Subsequently, promastigotes at a concentration of 2×10^6 parasites/ml (parasite/macrophage ratio 10:1) were seeded on the plate after the removal of the growth medium. The cells were infected by each individual strain or by mixed strains (i.e., *L. infantum* IPT1 + *L. tarentolae* LEM-124; *L. infantum* IPT1 + *L. tarentolae* RI-325), maintaining the same proportion of promastigotes/macrophage on all infection conditions, and the plate was incubated at 37°C with 5% CO_2. After 4 h of infection, each well was washed twice with sterile PBS to remove non-internalized parasites. The plates were then maintained to monitor the infection progression during post-infection time intervals (4–96 h). All experiments were conducted in triplicate. A negative non-infected control was included in each experimental assay.

Evaluation of *Leishmania* spp. intracellular parasite burden

For each time point, coverslips containing infected macrophages were subjected to Diff-Quick® staining (Merck, Darmstadt, Germany) following the established protocol previously described[48]. Intracellular parasites were monitored at different times post-infection (i.e. 4, 8, 24, 48, 72, and 96 h). Stained cells on coverslips were observed under bright-field microscopy at 100× magnification (LEICA DM LB2, Germany). The internalization capacity of parasites was determined by counting a total of 200 cells to estimate the percentage of infection and the number of amastigotes per infected cell (a/infc).

RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

The mechanically scraped cells from each infection time point were collected and immediately subjected to centrifugation at 12,000 × *g* for 10 min at RT. The resulting pellet was resuspended in 800 µL of Trizol (Invitrogen, USA) and stored at − 80 °C until RNA extraction. Scraped cells were submitted to RNA extraction by Trizol (Invitrogen, USA) accordingly to the manufacturer guidelines. The final pellet was eluted in 15 µL of DEPC (Diethyl pyrocarbonate)-treated water. The synthesis of cDNA was performed in 10 µL reverse transcription reactions using 3 µL of total RNA in 1× SuperScript IV VILO Mastermix (Thermo Fisher Scientific, Whaltam, USA). The RT-PCR synthesis was carried out at 25 °C for 10 min, followed by 50 °C for 10 min, and at 85 °C

for 5 min. The cDNA concentration was quantified by Qubit dsDNA HS Assay Kit and stored at – 20 °C. The quantitative PCR (qPCR) for cytokine gene expression of and anti- *IL-6*, *IL-10*, *IL-4* and pro-inflammatory *TNF-α*, *IL-12* were carried out with 10 µL of SsoAdvanced™ Universal Supermix (Bio-Rad, CA, USA) and 0.7 µL of PrimerPCR Costum Assay of primers (400 nM) and probes (250 nM) previously reported with modifications[49], (S1 Table). Similarly, gene expression of *IFN- γ* was also evaluated, being macrophages able to produce this cytokine, as elsewhere described[50]. The qPCR reactions were performed at 55 °C for 2 min, 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min for each cytokine. We monitored the expression of the control genes *G3PDH*[49] and *OAZ1*[51] for normalization purposes. Cytokine gene expression was assessed using the $2^{-\Delta\Delta Cq}$ value[52], by computing the ΔCq from the reference gene and the target gene in each sample. Subsequently, ΔΔCq was calculated by subtracting the ΔCq of the control group from the ΔCq of the experimental group. Gene expression levels were expressed as mRNA relative units corresponding to the ratio between the gene expression of infected and uninfected macrophages.

Statistical data

Statistical analyses were performed using GraphPad Prism v. 8.0.0 (GraphPad Software, San Diego, CA, USA). Shapiro–Wilk test was used to assess normal data distribution. Two-way ANOVA followed by Tukey's post hoc test was performed for the triplicates of each variable. Values were considered statistically significant when $P < 0.05$.

Data availability

All data analyzed during the study are included in this published article.

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Author contributions

V.N.L.F., M.S.L. and D.O. performed the conceptualization, investigation, supervision, project administration, visualization, and wrote the main manuscript text. V.N.L.F. performed the formal analysis, validation and software analysis. V.N.L.F. and M.S.L. performed data curation. V.N.L.F. and M.S.L. performed the methodology. D.O. was responsible for the funding acquisition. J.A.M.R., S.E., I.V.B. and C.B. reviewed and edited the manuscript. All authors reviewed the manuscript.

Competing interests
The authors declare no competing interests.

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