

Effects of tropism and virulence of *Leishmania* parasites on cytokine production by infected human monocytes

A. Meddeb-Garnaoui, H. Zrelli and
K. Dellagi

Laboratory of Immunology, Institut Pasteur de
Tunis, Tunis-Belvédère, Tunisia

Summary

The nature of early interactions between *Leishmania* and macrophages which determine the outcome of infection can be related directly to parasite biological properties. Here we compared the capacity of *L. major* (*Lm*) strains, reported to be high (*LmHV*) and low virulent and (*LmLV*) in the mouse model and *L. infantum* (*Li*) strains, dermatropic (*LiD*) and viscerotropic (*LiV*), to infect and modulate cytokine production in human peripheral blood derived monocytes. Monocytes were infected with metacyclic promastigotes for 24, 48 and 72 h. Parasite burden was significantly higher in *Lm*- than in *Li*-infected monocytes. *LmHV* and *LiD* induced a significantly higher parasite burden than *LmLV* and *LiV* respectively. Cytokine production was evaluated in monocytes infected for 24 h. Contrary to interleukin (IL)-12p70, monocyte chemotactic protein-1 and transforming growth factor- β production was increased significantly in infected monocytes with no differences between strains. *Lm* isolates induced significantly higher quantities of tumour necrosis factor (TNF)- α than *Li* isolates. Low levels of IL-10 were induced by all *Leishmania* strains and, interestingly, co-stimulation with lipopolysaccharide (LPS) was accompanied by a dramatic increase in IL-10 production by infected monocytes. In conclusion, *Lm* isolates displaying different levels of virulence in mice exhibited significant differences in parasite burden but similar abilities to modulate cytokine production in human monocytes. *Li* strains showed weaker infectivity and TNF- α inducing-capacity compared with *Lm* strains. The dramatic increase of IL-10 production in infected monocytes co-stimulated by LPS may play a role in disease progression considering the presence of LPS during bacterial superinfections observed during human leishmaniasis.

Keywords: cytokines, *Leishmania*, monocyte, tropism, virulence

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Correspondence: A. Meddeb-Garnaoui,

Laboratory of Immunology, Institut Pasteur
de Tunis, 13, Place Pasteur-BP74. 1002

Tunis-Belvédère, Tunisia.

E-mail: amel.garnaoui@pasteur.rns.tn.

Introduction

Leishmania are intracellular protozoa that cause a wide spectrum of human diseases, ranging from self-healing cutaneous to lethal visceral leishmaniasis (VL) [1]. In Tunisia, *L. major* (*Lm*) causes cutaneous leishmaniasis (CL) which heals spontaneously [2], whereas *L. infantum* (*Li*) is mainly responsible for VL, fatal if untreated, [3] and occasionally for a sporadic form of CL with chronic and disfiguring lesions [4]. These various clinical outcomes are influenced by both differences in *Leishmania* species and nature of the host cellular immune response. In mice, resistance to *Lm* infection is based on an interleukin (IL)-12-driven T helper type 1 (Th1) response and interferon (IFN)- γ production resulting in

macrophage activation and parasite killing. Development of Th2 cells with IL-10 and transforming growth factor (TGF)- β contribute to susceptibility by down-regulating Th1 cell differentiation and suppressing macrophage activity [5,6]. Information about the early interactions occurring between human monocytes or macrophages and *Leishmania* parasites is limited. These cells are the major site for parasite replication and, once a protective Th1 immune response is established, the principal effector cells responsible for their killing [7]. Among the mechanisms developed by *Leishmania* to promote their intracellular survival is the modulation of macrophage-associated cytokine production [8]. *Leishmania* promastigotes of different species are potent inhibitors of macrophage IL-12 production [9,10]. Tumour necrosis

factor (TNF)- α was not detected in monocytes infected with *L. donovani* amastigotes [11] or with *Lm* or *L. chagasi* promastigotes [12,13], whereas it was produced by *Lm* amastigote-infected macrophages [14]. *Leishmania* can also induce production of macrophage deactivating cytokines such as IL-10 [15,16] or TGF- β [17–19]. Monocyte chemoattractant protein-1 (MCP-1), a CC chemokine crucial in recruitment and activation of monocytes, is produced in *Lm*-infected human monocytes which stimulates the elimination of *Leishmania* by these cells directly [20]. *Leishmania*-mediated alterations of cytokine or chemokine production which influence the course of disease can vary depending on the *Leishmania* species or strain. Disease progression in BALB/c mice infected with different *Lm* strains isolated from human cutaneous lesions was heterogeneous, with differences observed in lesion size and some parameters of the immune response [21].

Here, we evaluated correlations between virulence or tropism of *Leishmania* strains and human monocyte response. We used two *Lm* strains showing different levels of virulence based on the severity of disease induced in BALB/c mice [21] and two *Li* strains, one viscerotropic and one dermatropic, to analyse infectivity and cytokine–chemokine production by human infected monocytes.

Materials and methods

Parasites

The *Lm* parasites isolated from human CL lesions [22] and *Li* parasites isolated from human sporadic CL lesions or from bone marrow aspirate of VL patients were used in this study. *Lm* isolates were selected on the basis of their experimental pathogenicity expressed in BALB/c mice: one high-virulence isolate (MHOM/TN/94/GLC94, identified as *LmHV*), inducing a severe disease, and one low-virulence isolate (MHOM/TN/94/GLC32, identified as *LmLV*) inducing a less severe disease [21]. *Li* were selected on the basis of their tropism: one viscerotropic isolate (MHOM/TN/98/LIPA843, identified as *LiV*) and one dermatropic isolate (MHOM/TN/99/LIPA922, identified as *LiD*). Parasites were cultivated on Novy–MacNeal–Nicolle medium at 26°C then adapted to RPMI-1640 (Sigma, St Louis, MO, USA) containing L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml) (RPMI PS/Glu) and 10% heat-inactivated fetal calf serum. Metacyclic promastigotes obtained by negative selection with peanut agglutinin were used for infection of human monocytes.

Preparation and culture of human monocytes

Blood donations were collected from healthy volunteers (who provided informed consent) at the Blood Transfusion Service of Tunis. Plasma was saved following centrifugation, and peripheral blood mononuclear cells (PBMCs) were

isolated by Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) gradient centrifugation, and resuspended to 2×10^6 /ml in RPMI PS/Glu supplemented with 10% fresh autologous plasma (AP). This suspension was added to gelatin/plasma-coated flasks and allowed to adhere for 2 h at 37°C, 5% CO₂, as described previously [23]. Non-adherent cells were removed and adherent cells were collected by scraping with a plastic cell scraper and washed. Purity of monocytes was > 80% and contained less than 3% T cells, as determined by flow cytometry using a fluorescence activated cell sorter (FACScan) flow cytometer (Becton Dickinson, San Jose, CA, USA) and staining with anti-CD14, anti-CD3 and anti-CD19 monoclonal antibodies (BD Biosciences Pharmingen, San Diego, CA, USA). Isotype-matched control antibodies were used for detecting non-specific binding to cells. Data were analysed using Lysis II software.

Parasite infection of human monocytes

Freshly purified monocytes were adjusted to 10^6 /ml in RPMI PS/Glu supplemented with 3% AP and distributed into plastic tissue culture dishes (eight-well Permanox chamber slides, Nunc, Naperville, IL, USA). Monocytes were incubated with metacyclic promastigotes, at a ratio of two parasites per cell, for 24 h at 37°C, 5% CO₂. Extracellular parasites were washed with warm phosphate-buffered saline (at 37°C), and cultures were incubated for a further 24 and 48 h. Monocytes were then stained with Giemsa and May–Grunwald solutions and analysed using light microscopy. Results were expressed as the percentage of infected monocytes and the mean number of intracellular parasites per infected monocyte. Enumeration was performed on 50 cells.

Cytokine assay

Monocytes were resuspended at 10^6 /ml in RPMI PS/Glu supplemented with 3% AP and distributed in 24-well plates. Monocytes were infected with metacyclic promastigotes, at a ratio of two parasites per cell, in the presence or absence of lipopolysaccharide (LPS) (1 μ g/ml) or incubated overnight with recombinant human IFN- γ (100 ng/ml) (BD Biosciences Pharmingen) and then co-incubated with promastigotes, at a ratio of two parasites per cell, and LPS (1 μ g/ml). Supernatants were taken 24 h later and stored at –80°C. Levels of IL-10, TGF- β , IL-12p40 and p70, TNF- α and MCP-1 were measured using the enzyme-linked immunosorbent assay sandwich method [24]. Purified and biotinylated antibodies specific for these cytokines and human recombinant cytokines used as standards were purchased from BD Biosciences Pharmingen. The thresholds of cytokines detected were 13.6 pg/ml for IL-12p70 and p40; 4.5 pg/ml for TNF- α and IL-10; 3.9 pg/ml for MCP-1; and 41 pg/ml for TGF- β .

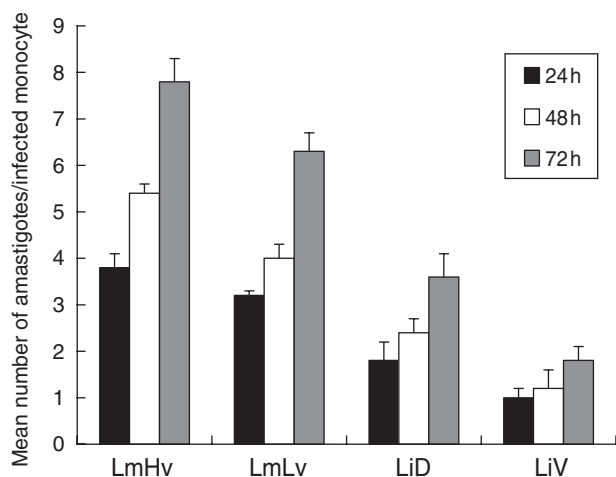


Fig. 1. Intracellular multiplication of *Leishmania major* and *L. infantum* amastigotes in human monocytes. Results are mean \pm standard deviation determined in three independent experiments.

Statistical analysis

Results are expressed as mean \pm standard error of the mean of three, four or five independent experiments. Statistical significance between treated and control cultures was analysed by Mann–Whitney *U*-test using Statview statistical software (version 5.0, 1998; SAS Institute Inc., San Diego, CA, USA). *P* values of $P < 0.05$ were considered significant.

Results

Human monocyte infection with *L. major* and *L. infantum* metacyclic promastigotes

Infection rates obtained with the two *Lm* isolates (100%) were similar and higher than those of *Li* isolates. The rate of infection was slightly higher for the dermatotropic isolate *LiD* (average of 69%) compared with the viscerotropic isolate, *LiV* (average of 57%). Over the 3-day period, the number of intracellular parasites doubled for all isolates (Fig. 1). At 48 and 72 h post-infection, the mean number of intracellular parasites was significantly higher in monocytes infected with *LmHV* than with *LmLV* and was twice as high with *LiD* isolate compared with *LiV* isolate ($P < 0.05$). Parasite burden was twice as high in *Lm*- than in *LiD*-infected monocytes and threefold higher than in *LiV*-infected monocytes, and these differences were statistically significant ($P < 0.05$) (Fig. 1).

Species-specific cytokine induction by *Leishmania*-infected monocytes

Monocytes from five donors were exposed to metacyclic *Lm* and *Li* promastigotes. After 24-h culture, supernatants were

collected and levels of IL-12p40, IL-12p70, IL-10, TNF- α , TGF- β cytokines and MCP-1 chemokine were analysed (Fig. 2). Both *Lm* isolates were able to induce significantly higher levels of TNF- α (897.5 pg/ml \pm 161.8 for *LmHV* and 940 pg/ml \pm 211.2 for *LmLV*), compared with *Li* isolates (285 pg/ml \pm 111.5 for *LiD* and 307.5 pg/ml \pm 98.1 for *LiV*, $P < 0.05$). No IL-12p70 was detected. However, unlike *Li* isolates, both *Lm* isolates induced low levels of IL-12p40. Significantly high levels of MCP-1, TGF- β and IL-10 were detected after stimulation with *Lm* or *Li* promastigotes, in comparison with uninfected cells. However, no significant difference in levels of these cytokines was observed between *LmHV* and *LmLV* or *LiV* and *LiD*.

Effect of IFN- γ priming on cytokine-inducing capacity of the *Leishmania* isolates in infected monocytes

To determine whether IFN- γ priming could influence cytokine-inducing capacity of the *Leishmania* isolates, used as described previously [10], and whether this property could vary in accordance to tropism or virulence, monocytes were first primed with IFN- γ for 12 h and then infected with parasites (Fig. 3). A significant increase of TNF- α production was observed as a consequence of IFN- γ priming. This increase was fivefold greater for *Li*-infected monocytes compared with *Lm*-infected monocytes (twofold increase). However, no differences were observed in accordance to virulence or tropism. IFN- γ priming induced a weak increase in IL-12p40 production in infected monocytes (Fig. 3), but was inefficient in inducing IL-12p70 production (data not shown). Similar results were observed for all *Leishmania* isolates tested. Finally, preincubation with IFN- γ inhibited IL-10 and TGF- β production drastically in monocytes infected with all *Leishmania* isolates tested. The inhibitory rate varied from 70 to 90%.

Enhancing effect of LPS on *Leishmania*-induced IL-10 production by human monocytes

Leishmania parasites can modulate the ability of macrophages to produce cytokines in response to strong proinflammatory stimuli. The presence of LPS is required for the induction of IL-10 by *Leishmania* in murine macrophages [15]. *Leishmania* inhibit IL-12 production induced by IFN- γ /LPS stimulation in murine macrophages and human PBMC [9,10,25]. To investigate whether the capacity to modulate macrophage-associated cytokine production in response to LPS varies depending on virulence or tropism of *Leishmania* strains, monocytes were incubated overnight with or without IFN- γ then co-incubated with parasites and LPS for 2 h to analyse IL-12 and IL-10 production. Interestingly, in accordance with previous data obtained in mice, LPS had a powerful enhancing effect on *Leishmania*-induced IL-10 production by human monocytes (Fig. 4). IL-10 production in monocytes co-stimulated with LPS and *Leishma*

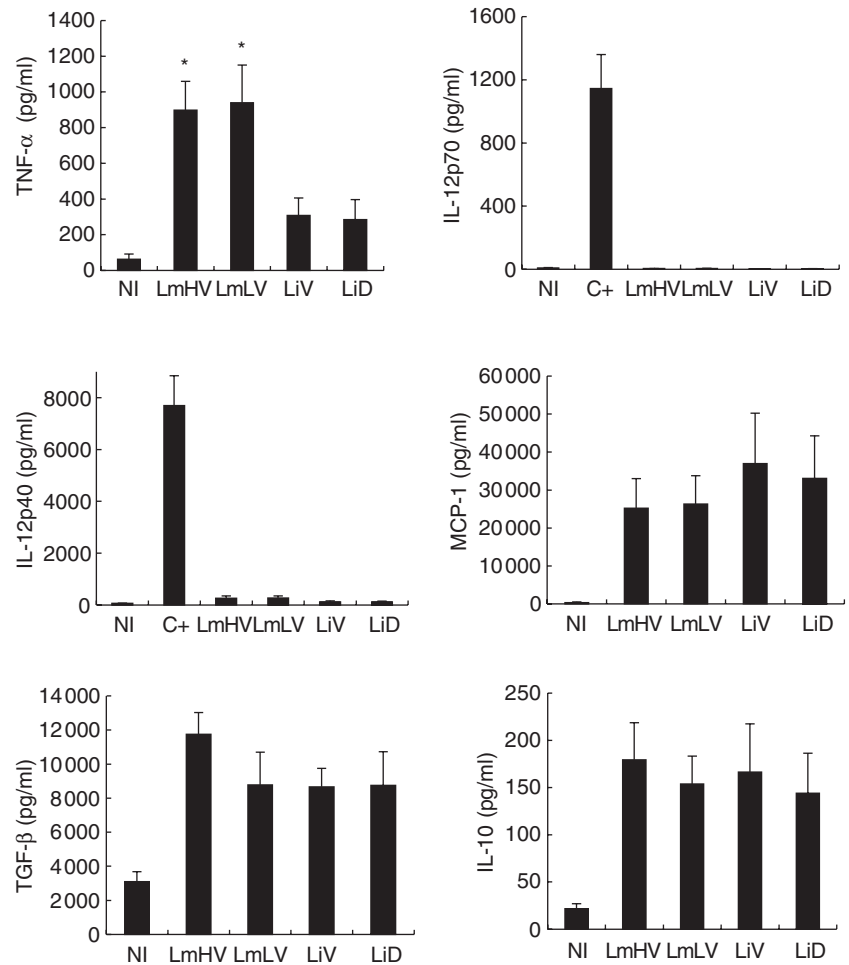


Fig. 2. Comparative analysis of cytokine or chemokine patterns induced by *Leishmania major* and *L. infantum* isolates in human monocytes. Monocytes were infected with metacyclic promastigotes derived from each of the four isolates, at a ratio of two parasites per cell. Culture supernatants were collected after 24 h and cytokines were quantitated by enzyme-linked immunosorbent assay (ELISA). Control cultures were stimulated with interferon- γ and lipopolysaccharide (C+). Results are mean \pm standard error of the mean of four or five experiments. $P < 0.05$ in comparison with non-infected (NI) cultures, except for interleukin (IL)-12p70 induced by the four isolates and for IL-12p40 induced by *Leishmania viscerotropic* (LiV) and *Leishmania dermatropic* (LiD) isolates. * $P < 0.05$ compared with LiV and LiD.

nia was 39–45-fold greater than in *Leishmania*-infected monocytes. No significant differences were observed, however, in accordance with virulence or tropism. Furthermore, and as shown previously [9,10,25], *Leishmania* inhibit IFN- γ /LPS induced-IL-12 p40 and p70 production (Fig. 5), but no significant difference was observed between studied isolates.

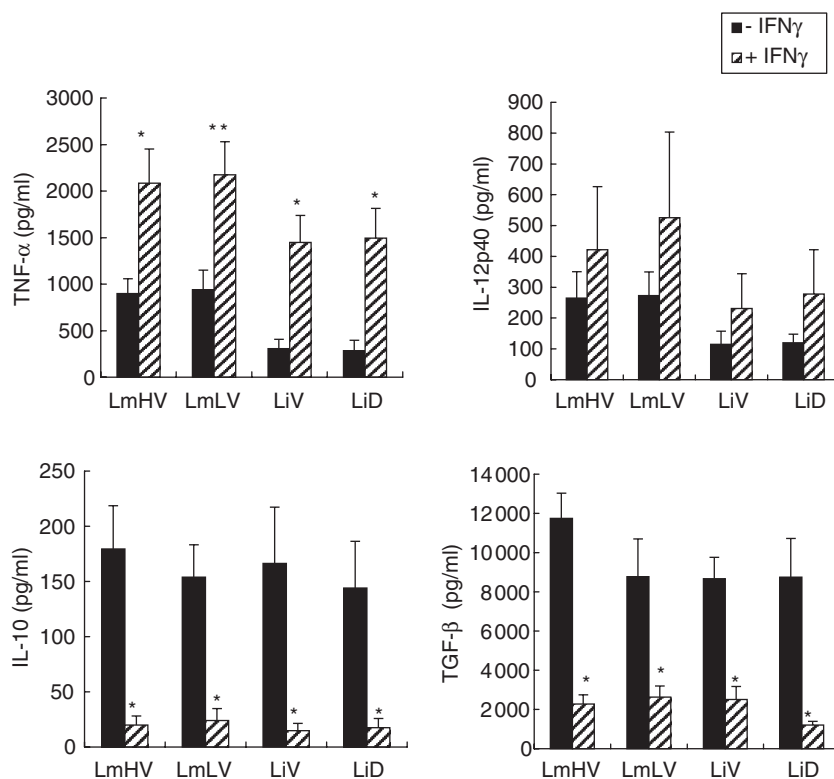
Discussion

To evaluate the impact of virulence and tropism of *Leishmania* isolates on infectivity and cytokine/chemokine production by human monocytes, we selected two *L. major* strains showing different levels of virulence in BALB/c mice [21] and two *L. infantum* strains, one viscerotropic isolated from a VL patient and one dermatropic isolated from a patient with sporadic form of CL. Monocyte infection rates were similar for *Lm* strains. However, a significantly higher parasite burden was observed in monocytes infected with *LmHV*. These results suggest that although *LmHV* and *LmLV* are ingested similarly by monocytes, *LmHV* replicates more rapidly inside the phagolysosome. While correlation between virulence of *Leishmania* strains and ability to survive in

murine macrophages has already been reported [21,26–28], much less is known about the human macrophage response. It was shown that unlike the wild-type parasite, an avirulent *Lm* mutant deleted for an essential metabolic gene (*DHFR-TS*) was phagocytized but unable to replicate in human macrophages [29]. For *Li*, the parasite burden in monocytes infected with *LiD* was significantly higher than in those infected with *LiV*. Moreover, infection rates were lower compared with those observed for *Lm* strains. Recently, Campos-Ponce *et al.* found no association between *Li* tropism (cutaneous versus visceral) and murine macrophage infection rates [30], whereas others showed higher infectivity for a visceral form compared with a cutaneous form [31]. *Li* tropism and hence virulence is certainly influenced largely by the host immune status as well as by strain genotype [32], and a variability within dermatropic or viscerotropic *Li* strains used could explain discrepancies observed.

To survive the hostile environment of macrophages, *Leishmania* developed subversion mechanisms including the modulation of cytokine production. Here, TNF- α levels were significantly higher for *Lm*- compared with *Li*-infected monocytes. We cannot exclude that differences in TNF- α production could be due to different rates of infection

Fig. 3. Comparative effect of interferon (IFN)- γ priming on cytokine production, in human monocytes infected by *Leishmania* parasites of different virulence and tropism. Monocytes were primed with IFN- γ for 12 h then infected with metacyclic *Leishmania* parasites for 24 h. Cytokines were quantified by enzyme-linked immunosorbent assay in culture supernatants. Results are expressed as mean cytokine production \pm standard error of the mean determined from five experiments for interleukin (IL)-12p40 and IL-10, four experiments for tumour necrosis factor- α and three experiments for transforming growth factor- β . * $P < 0.05$; ** $P = 0.05$ in comparison with unprimed monocytes.



observed between *Lm* and *Li* strains. No correlations were found with virulence or tropism of *Leishmania* isolates, although data in murine models indicate that implication of TNF- α in the control of *Leishmania* infection seems to depend on parasite strain [33]. Variability in TNF- α -inducing capacity among *Leishmania* species has been shown previously in murine models [34,35]. In human, some studies demonstrated that *Leishmania* was able to infect monocytes without inducing TNF- α production [11–13], whereas others report the opposite [14]. In agreement with previous studies [11,14], we observed a significant increase of TNF- α production as a consequence of IFN- γ priming. This increase was greater in *Lm* than in *Li*-infected monocytes. These data, adding to the role of TNF- α in enhancing leishmanicidal activity in IFN- γ -activated macrophages [36], argue that TNF- α modulation by *Leishmania* during the early inflammatory events may be associated directly with the outcome of disease.

We showed that *Leishmania* strains did not induce IL-12 production, and inhibited the IFN- γ /LPS-induced IL-12 production. Furthermore, IFN- γ priming was inefficient to overcome the inhibition of IL-12 production by *Leishmania* isolates. However, no correlation was found between these response profiles and virulence or tropism. Similarly, the absence of IL-12 in supernatants of virulent or avirulent *Lm*-infected human macrophages was reported [29]. It was also shown that *Lm* and *L. amazonensis* were unable to

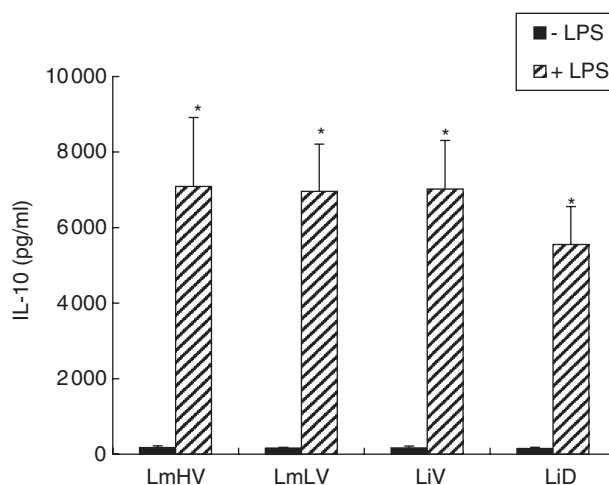


Fig. 4. Comparative analysis of interleukin (IL)-10 production induced by *Leishmania* isolates differing by virulence or tropism, in lipopolysaccharide (LPS)-activated monocytes. Monocytes were treated with LPS (1 μ g/ml) and infected simultaneously with *Leishmania* parasites for 24 h. IL-10 was quantified in culture supernatants by enzyme-linked immunosorbent assay. Results are expressed as mean cytokine production \pm standard error of the mean determined from five experiments. * $P \leq 0.01$ in comparison with *Leishmania*-activated monocytes. Control cultures were monocytes stimulated with LPS (mean of IL-10 production 1965 pg/ml \pm 318.6).

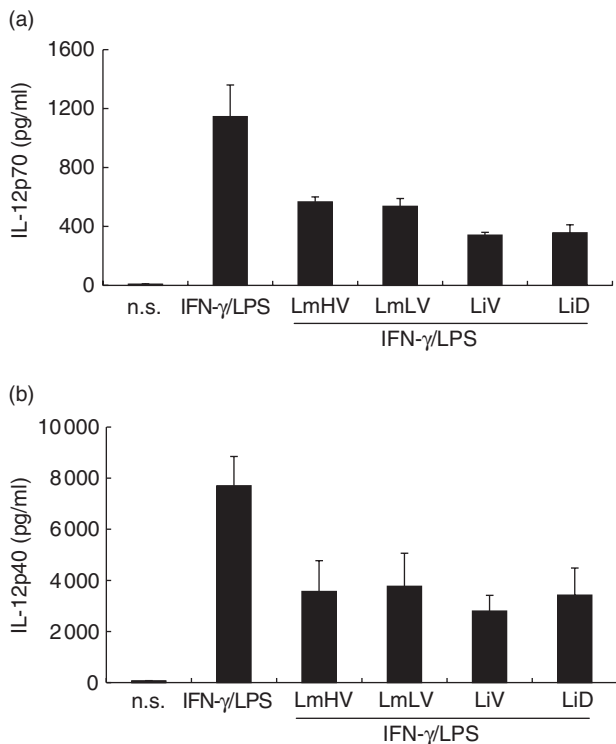


Fig. 5. Effect of virulence or tropism of *Leishmania major* and *L. infantum* isolates on interleukin (IFN)- γ /lipopolysaccharide (LPS)-induced production of interleukin (IL)-12 after infection of human monocytes. Monocytes were primed for 12 h with IFN- γ then co-stimulated for 24 h with *Leishmania* parasites and LPS. IL-12p70 (a) and IL-12p40 (b) were then quantified in culture supernatants. Results are mean \pm standard error of the mean of three experiments; n.s.: non-stimulated and non-infected cultures.

induce IL-12 production in murine peritoneal macrophages *in vitro* and IFN- γ priming had no effect on IL-12 levels [37]. Adding to our results, all these data suggest that virulence of *Leishmania* does not influence the inhibition of IL-12 production by monocytes or macrophages.

We showed a pronounced induction of MCP-1 expression by *Lm*, as shown previously [12,20]. Similar results were obtained for *Li*-infected monocytes. MCP-1 was implicated in the recruitment of monocytes and the stimulation of macrophage leishmanicidal functions and its pattern of expression was correlated with the type of cutaneous disease [20,38]. In murine macrophages, MCP-1 expression seems to depend on the virulence of *Leishmania* strains [26]. These discrepancies could be explained by the different systems used (human *versus* mouse) or by comparison of virulent *versus* avirulent strains [26] *versus* *Lm* isolates which exhibit variable levels of virulence (our study).

Lm and *Li* isolates were able to induce significantly elevated levels of IL-10 in human monocytes regardless of their virulence or tropism. On the contrary, using *Lm*, *L. chagasi* or *L. amazonensis*, others found that parasite infection does not induce IL-10 release in human PBMC [10] or

monocytes [13]. Despite the fact that we did not detect any significant association between IL-10-inducing capacity and virulence of *Lm* or *Li* isolates, discrepancies observed between our results and literature data can be attributed to variability in *Leishmania* strains or species. In addition, these different results may be due to the use of different cell populations, PBMC or macrophages or monocytes. It was suggested that the absence of IL-10 induction could be explained by the fact that optimal IL-10 production by macrophages probably depends on Fc γ R ligation by surface IgG [15]. However, our results and others, showing that *in vitro* infection of murine macrophages with non-opsonized *L. donovani* promastigotes caused significant induction of IL-10 release, suggest that other mechanisms may contribute to IL-10 induction in macrophages [39]. Interestingly, we also observed that infection had a powerful enhancing effect on LPS-induced IL-10 production by human monocytes. On the contrary, it was shown that IL-10 production by LPS-stimulated *L. chagasi*-infected monocytes was similar to uninfected controls [13]. However, IL-10 induction in mice by *Leishmania* amastigotes required the presence of LPS [15]. Considering the presence of LPS during bacterial superinfections that can be observed during visceral and CL [40,41], it is tempting to speculate that the simultaneous presence of LPS and parasites could induce a robust production of IL-10 that may play a role in progression of disease.

Finally, significantly elevated levels of TGF- β were detected following stimulation with all *Leishmania* strains, but no correlation with virulence or tropism was observed. A direct correlation between the amounts of TGF- β produced following *in vitro* infection of murine macrophages and virulence of two *L. braziliensis* isolates was demonstrated [17]. It was also reported that *Li* strains with the higher infectivity induce higher levels of TGF- β in murine macrophages [28]. These discrepancies can be attributed to the use of human *versus* mouse models and point out once again the difficulties of relating results observed in experimental murine leishmaniasis to human leishmaniasis.

In conclusion, we showed in this study that *Lm* strains displaying different levels of virulence in BALB/c mice and viscerotropic and dermatropic *Li* strains displayed similar MCP-1-, IL-10- and TGF- β -inducing capacities as well as similar abilities to inhibit IL-12 production in human monocytes, despite significant differences in infectivity. Such results obtained *in vitro*, where several factors playing an important role in *in vivo* *Leishmania*-macrophage infection are absent, do not exclude an implication of parasite virulence in the innate immune response of the human host, as shown in the mouse model. Nevertheless, our results reveal that *Li* are weaker TNF- α inducers than *Lm* in human monocytes, indicating that TNF- α modulation by *Leishmania*, during the early inflammatory events, may be associated directly with the type of species-specific pathogenesis that will develop later.

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