

# *Leishmania amazonensis* infection impairs differentiation and function of human dendritic cells

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**Abstract:** Dendritic cells (DCs) are of utmost importance in initiating an immune response and may also function as targets for pathogens. The presence of pathogens inside DCs is likely to impair their functions and thus, influence immune responses. In the present report, we evaluated the impact of the presence of *Leishmania amazonensis* during differentiation and maturation of human monocyte-derived DCs. The presence of live *L. amazonensis* parasites during DC differentiation led to a significant decrease in CD80 (92%) and CD1a (56%) expression and an increase in CD86 (56%) cell surface expression. Phenotypic changes were accompanied by a lower secretion of IL-6, observed after 6 days of DC differentiation in the presence of *L. amazonensis*. DCs differentiated in the presence of *L. amazonensis* were used as APC in an autologous coculture, and lower amounts of IFN- $\gamma$  were obtained compared with control DCs differentiated in the absence of parasites. The effect of heat-killed parasites, but not of *Leishmania* antigen, during DC differentiation and maturation was similar to that observed with viable parasites. During maturation, the presence of live *L. amazonensis* parasites, but not of soluble *Leishmania* antigen, led to a decrease in IL-6 and IL-10 production. In this way, we observed that the parasite is able to abrogate full DC differentiation, causing a delay in the immune response and likely, favoring its establishment in human hosts. *J. Leukoc. Biol.* 82: 1401–1406; 2007.

**Key Words:** *Leishmaniasis* · antigen-presenting cells · cytokines · costimulatory molecules · T cells

## INTRODUCTION

Dendritic cells (DCs) are highly specialized APC, which receive incoming signals from innate immunity and convert them to an adequate T cell response [1]. DCs are essential for an effective immune response against pathogens, which in turn, have developed mechanisms to avoid initiation of immune responses, for example, by interfering with DC biology and function. Several pathogens, such as HIV [2], *Plasmodium*

*falciparum* [3], and *Brugia malayi* microfilaria [4], alter DC function and impair efficient, protective immune responses. Mechanisms described already for these pathogens include inhibition of expression of costimulatory molecules during LPS-induced maturation by *P. falciparum* [3] or down-modulation of IL-12 and IL-10 after maturation, as described for *B. malayi* microfilaria [4]. DCs on peripheral regions are in an immature state and have maximal antigen-uptake capacity. Once activated, DCs produce IL-12, which promotes Th1 differentiation, characterized by IFN- $\gamma$  production. This cytokine is critical for the killing activity of *Leishmania*-infected macrophages. The secretion of anti-inflammatory cytokines such as IL-10 can also preclude immunopathological reactions [5]. Recent studies suggest that *Leishmania amazonensis* can infect and possibly alter DC biology, favoring the establishment of infection. Studies in mouse models have demonstrated that amastigotes and metacyclic parasites enter and activate DCs efficiently. However, infection with amastigotes fails to induce CD40-dependent IL-12 production, and there is IL-4 production in BALB/c DCs, conditioning a Th2 priming [6].

It has been shown that DCs infected with *Leishmania infantum* amastigotes are able to mature when stimulated with LPS [7]. It is interesting that there is no data about the interaction of *L. amazonensis* with human DCs. *L. amazonensis* is responsible for diffuse cutaneous leishmaniasis (DCL), a severe, clinical manifestation characterized by a high number of parasites and a lack of T cell response [8]. In the present study, we examined the role of *L. amazonensis* during in vitro differentiation and maturation of human DCs to better understand the initial steps of the induction of human anti-*Leishmania* immune responses.

## MATERIALS AND METHODS

### Parasites

*L. amazonensis* (MHOM/BR/87/BA125) promastigotes were grown at 23°C in Schneider's *Drosophila* medium (Sigma-Aldrich, St. Louis, MO, USA) contain-

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ing 5% heat-inactivated FBS (Cripion Biotechnology, Brazil). Metacyclic promastigotes were obtained from stationary-phase cultures (5–7 days) with less than five *in vitro* passages and then used to perform cell infection. Parasites were incubated at 65°C for 30 min to prepare heat-killed *Leishmania*. Heat killing was confirmed by the absence of a parasite growing in Schneider's *Drosophila* medium (Sigma-Aldrich).

## Antigen

Soluble *Leishmania* antigen (SLA) was prepared as described elsewhere [9]. Briefly, *L. amazonensis* stationary-phase promastigotes were sonicated and centrifuged at 20,000 *g* for 2 h. The supernatant was used at a final concentration of 10 or 50 µg/ml.

## In vitro generation of DCs

Immature, monocyte-derived DCs were prepared from peripheral blood monocytes obtained from healthy donors (Hemoba, Salvador/Bahia/Brazil). Briefly, PBMC were obtained from heparinized venous blood by passage over a Ficoll Hypaque gradient (Sigma-Aldrich). PBMC were washed three times, and the CD14<sup>+</sup> cell population was enriched by positive selection using magnetic cell sorting (Mini Macs, Miltenyi Biotec, Auburn, CA, USA). Monocytes were resuspended at a concentration of  $5 \times 10^5$  cells/ml in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 2 mM L glutamine, penicilin (100 U/ml), streptomycin (100 µg/ml; Gibco), and 10% heat-inactivated FBS (Cripion Biotechnology), plus IL-4 (100 U/ml) and GM-CSF (50 ng/ml; PeproTech, Rocky Hill, NJ, USA). Cells were plated in 24-well tissue-culture plates (Costar, Corning, NY, USA) and incubated at 37°C 5% CO<sub>2</sub> for 7 days. SLA (50 µg/ml) or *L. amazonensis* (10:1 *Leishmania*:DC ratio, without any washing to remove free parasites) was added on the same days as IL-4 and GM-CSF to study the effect of parasites or SLA on differentiation. At Days 3 and 6, fresh medium was replaced with GM-CSF and IL-4 without further addition of parasites. After 7 days, to characterize the DC population, cells were stained with anti-CD1a (PharMingen, San Diego, CA, USA), and fluorescence was analyzed by FACS (FACSort, Becton Dickinson, San Jose, CA, USA). Cultures contained more than 80% CD1a-positive cells, which were harvested, washed twice with saline, and used in different phenotypic and functional experiments.

## In vitro maturation of DCs

On Day 7 of culture, DCs were harvested and cultured at  $5 \times 10^5$ /ml in a 24-well tissue-culture plate in RPMI-1640 medium (Gibco), plus 10% heat-inactivated FBS (Cripion Biotechnology; supplemented medium), or in supplemented medium containing TNF-α [25 ng/ml, Sigma-Aldrich; complete medium (CM)]. Supernatants and cells were collected after 48 h for cytokine measurement and phenotypical/functional assays, respectively.

## Flow cytometry

After culture, immature and mature DCs were harvested for flow cytometric analyses. Briefly,  $5 \times 10^5$  cells were incubated with PBS/1% BSA (Sigma-Aldrich)/0.1% sodium azide (Nuclear) and incubated with 20% FCS to block FeR [10]. Cells were stained with PE-conjugated anti-CD1a (HI149), -CD80 (3H5), -CD83 (HB15A), and -CD86 (IT2.2), HLA-DR (L249), and DC-specific ICAM-grabbing nonintegrin [SIGN; (DCN46) all from BD Biosciences (San Jose, CA, USA); and anti-CD83, from Immunotech (France)]. All analyses included the appropriate isotype controls. Cells were analyzed on FACSort (Becton Dickinson) and CellQuest programs (Becton Dickinson).

## T cell isolation

PBMC were obtained from healthy donors (Hemoba), as described above. Cells were washed three times in saline solution, adjusted to  $5 \times 10^6$ /ml in RPMI medium, and plated in 24-well culture plates. After 30 min at 37°C, 5% CO<sub>2</sub>, nonadherent cells were obtained and used for an allogeneic MLR.

## Allogeneic MLR

Nonadherent cells from an allogeneic donor (100,000) were cultured in 96-well U-bottom microplates with 10,000, 2000, or 1000 immature or mature DCs, which were irradiated at 4000 rad (IBL 437 C, CIS Bio International, France) before performing MLR. [<sup>3</sup>H]Thymidine incorporation was measured on Day 4

after an 18-h pulse with [<sup>3</sup>H]thymidine solution (1 µCi/ml). Incorporation of radioactive label was measured by liquid scintillation (Cell Harvester, Filter-Mate 196, PerkinElmer Life Sciences, Boston, MA, USA). Results are expressed as the mean cpm of triplicate cultures (Direct Beta Counter, Matrix 9600, PerkinElmer Life Sciences).

## Autologous cocultures

Autologous T cell response to *Leishmania* was performed as described [11] with some modifications. Briefly, DCs were obtained from healthy donors and were used as APC. DCs were differentiated as described above. After 7 days, following a new blood collection, nonadherent cells were obtained from PBMC from the same donors, and a coculture was performed with monocyte-derived DCs, which had differentiated previously in the presence or absence of parasites. Cells were stimulated with *Leishmania* parasites (10:1 *Leishmania*:DC ratio) or SLA (10 µg/ml). After 72 h of coculture, supernatants were harvested for cytokine measurement (ELISA), and nonadherent cells were stained for flow cytometry analyses.

## Cytokine assays

All cytokines (TNF-α, IL-10, IL-6, IL-12, and IFN-γ) were detected on culture supernatants using commercially available ELISA kits from BD Biosciences and Nunc (Rochester, NY, USA) Maxisorp plates. Recombinant cytokines were used to obtain standard curves to calculate cytokine concentration in the supernatants.

## Statistical analysis

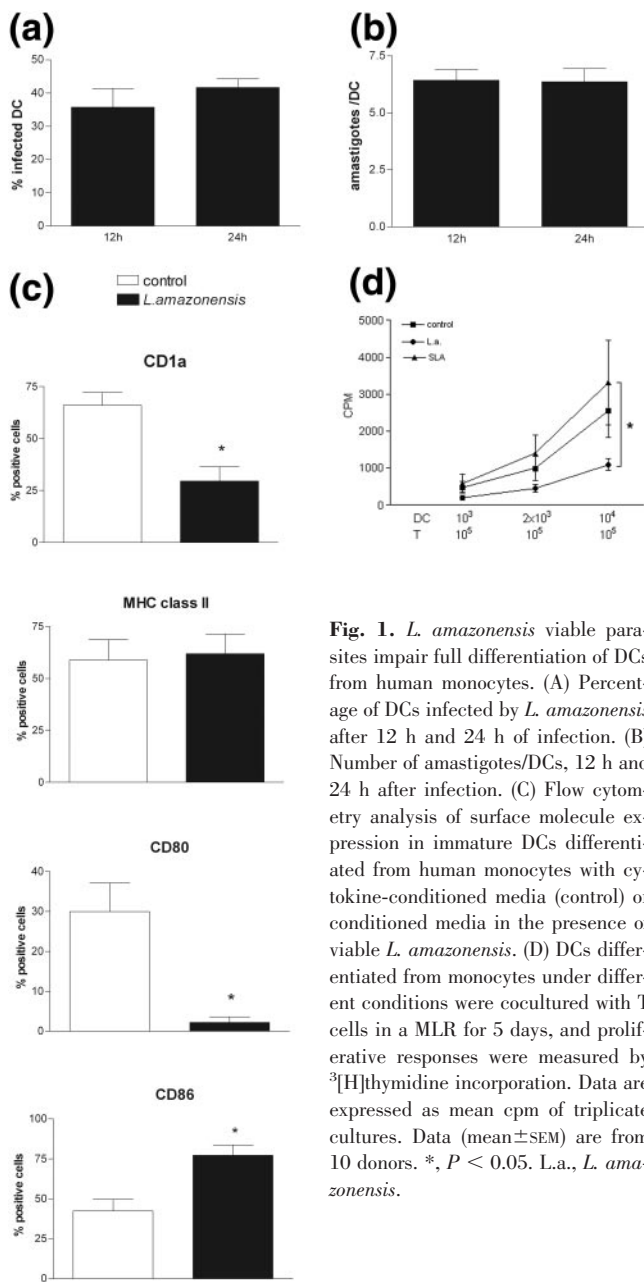
Data were analyzed using GraphPad Prism 4.0 (San Diego, CA, USA). Results were expressed as the mean ± SEM, as analyzed by one-way ANOVA tests with Bonferroni-Dunn post-tests; a *P* < 0.05 was considered significant.

## RESULTS

### *L. amazonensis* alters DC differentiation

We first asked whether *Leishmania* or SLA could interfere with the differentiation of immature DCs from human peripheral blood monocytes *in vitro*. Initially, we evaluated whether *L. amazonensis* could infect human DCs. Data from infection showed that *L. amazonensis* was able to infect human DCs, with low variation among donors (**Fig. 1A**) and also low variation in parasite burden with  $6.4 \pm 0.5$  amastigotes/DC after 24 h of infection (**Fig. 1B**). Then, we differentiated DCs from monocytes in the presence of SLA or in the presence of stationary-phase *L. amazonensis* promastigotes. DC differentiation was not completed when monocytes were cocultured with live *L. amazonensis* parasites. There was a diminished expression of CD1a and CD80 (**Fig. 1C**, *P* < 0.05) on DC cell surface when compared with control DCs, differentiated in the absence of parasites. It is interesting that expression of CD86 was higher (*P* < 0.05) in DCs differentiated in the presence of parasites. Conversely, monocytes were able to fully differentiate into immature DCs in the presence of SLA (data not shown).

Next, we investigated whether DCs cocultured with live parasites or SLA altered T cell proliferation. DCs differentiated in the presence of live *L. amazonensis* induced a significantly less-intense MLR, more evident at a 1:10 (DC:T) ratio when compared with SLA-cocultured DCs (**Fig. 1D**, *P* < 0.05). Such findings are in accordance with the surface molecule expression profile of *L. amazonensis*-exposed DCs, indicating that monocyte-derived DCs obtained under these conditions showed a decreased ability as APC. Last, we compared heat-killed and live *L. amazonensis* in DC differentiation. Whole



**Fig. 1.** *L. amazonensis* viable parasites impair full differentiation of DCs from human monocytes. (A) Percentage of DCs infected by *L. amazonensis* after 12 h and 24 h of infection. (B) Number of amastigotes/DCs, 12 h and 24 h after infection. (C) Flow cytometry analysis of surface molecule expression in immature DCs differentiated from human monocytes with cytokine-conditioned media (control) or conditioned media in the presence of viable *L. amazonensis*. (D) DCs differentiated from monocytes under different conditions were cocultured with T cells in a MLR for 5 days, and proliferative responses were measured by <sup>3</sup>H]thymidine incorporation. Data are expressed as mean cpm of triplicate cultures. Data (mean±SEM) are from 10 donors. \*,  $P < 0.05$ . L.a., *L. amazonensis*.

parasites led to similar results, regardless of viability (data not shown).

### IL-6 is involved in the incomplete differentiation of monocyte-derived DCs

Results showed that only the presence of whole *L. amazonensis* parasites altered DC cell surface molecule expression. Therefore, we asked whether cytokines could be involved in this process. No detectable levels of IL-10 and IL-12 were found in the supernatants of monocyte-derived DCs in the presence or absence of *Leishmania*. Although we obtained detectable levels of TNF- $\alpha$  in culture supernatants, there was no difference in its amount between DCs differentiated in the presence or absence of the parasite (data not shown). As shown in **Figure 2**, IL-6 production remained unaltered on the 3rd day of culture, but there was a significant reduction in its level on the 6th day of

culture in DCs differentiated in the presence of *L. amazonensis* (Fig. 2,  $P=0.0073$ ).

### DCs differentiated with *L. amazonensis* induced lower production of IFN- $\gamma$ by nonadherent cells in an autologous coculture

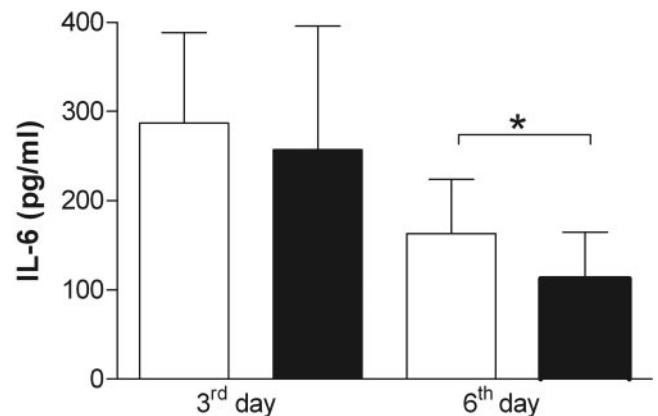
We observed an altered pattern of costimulatory molecule expression and IL-6 production on DCs differentiated in the presence of *L. amazonensis*. Therefore, our next question was to investigate whether these differences could affect specific *Leishmania* T cell responses. Knowing the importance of IFN- $\gamma$  induction in protective immune responses to *Leishmania*, we measured this cytokine in coculture experiments (see Materials and Methods), and we observed that monocyte-derived DCs, in the presence of *L. amazonensis*, induced significantly lower IFN- $\gamma$  production than control DCs (**Fig. 3**,  $P<0.05$ ).

### Maturation of DCs is not affected by *L. amazonensis* or by SLA

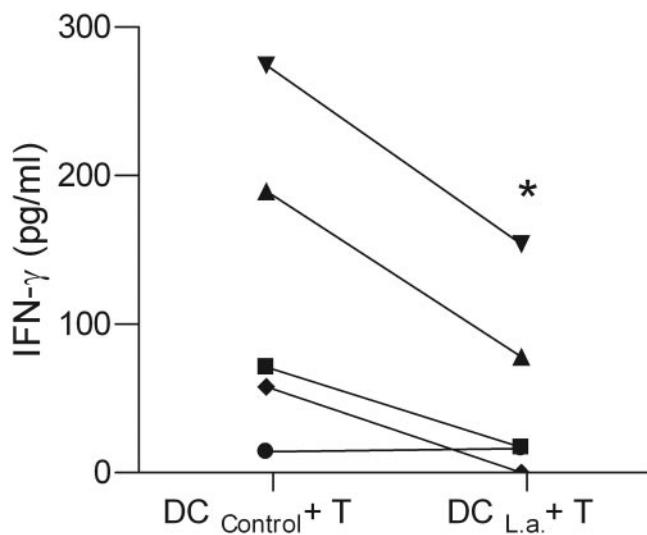
As *L. amazonensis* affected the differentiation of monocytes into DCs, the next question was to verify whether the DC maturation process could also be altered by the parasite. DCs were differentiated from monocytes in the presence of IL-4 plus GM-CSF for 7 days and were matured in the presence of TNF- $\alpha$  for 48 h. *L. amazonensis* or SLA was added to the culture simultaneously with TNF- $\alpha$ . Maturation was confirmed by up-regulation of costimulatory molecule expression, mainly CD83 (**Fig. 4A**). TNF- $\alpha$  added concomitantly with *L. amazonensis* (Fig. 4B) or SLA (data not shown) did not alter CD80, CD83, CD86, MHC class II, and DC-SIGN surface expression. Functional analysis also confirmed that DCs underwent full maturation despite *L. amazonensis* or SLA presence, as no significant differences on MLR were observed (Fig. 4C).

### Cytokine production during maturation of DCs is diminished by *L. amazonensis*

Although we did not observe significant differences in surface molecule expression or in MLR when DCs were matured in the



**Fig. 2.** *L. amazonensis* parasites inhibit IL-6 production during DC differentiation. Cytokine levels measured on culture supernatants on the 3rd and 6th days during DC differentiation from human monocytes. Control DCs were cultured in cytokine conditioned media only (open bars) or treated with viable *L. amazonensis* parasites (solid bars). Data (mean±SEM) are from 10 donors. \*,  $P < 0.05$ .



**Fig. 3.** IFN- $\gamma$  production is diminished significantly during coculture of DCs and T cells. DCs, differentiated with cytokine conditioned media (control) or in the presence of *L. amazonensis* parasites, were cocultured with T cells in an autologous leukocyte reaction for 3 days. IFN- $\gamma$  levels were detected by ELISA in the supernatants of these cultures. Data are from five individual donors. \*,  $P < 0.05$ .

presence of *L. amazonensis*, we evaluated whether cytokine production was modified under these same conditions. In this case, the presence of *L. amazonensis* parasites during the maturation process induced lower amounts of IL-6, IL-10, and IL-12 in culture supernatants when compared with control DCs (matured in the absence of parasites; **Fig. 5C**;  $P < 0.05$ ).

## DISCUSSION

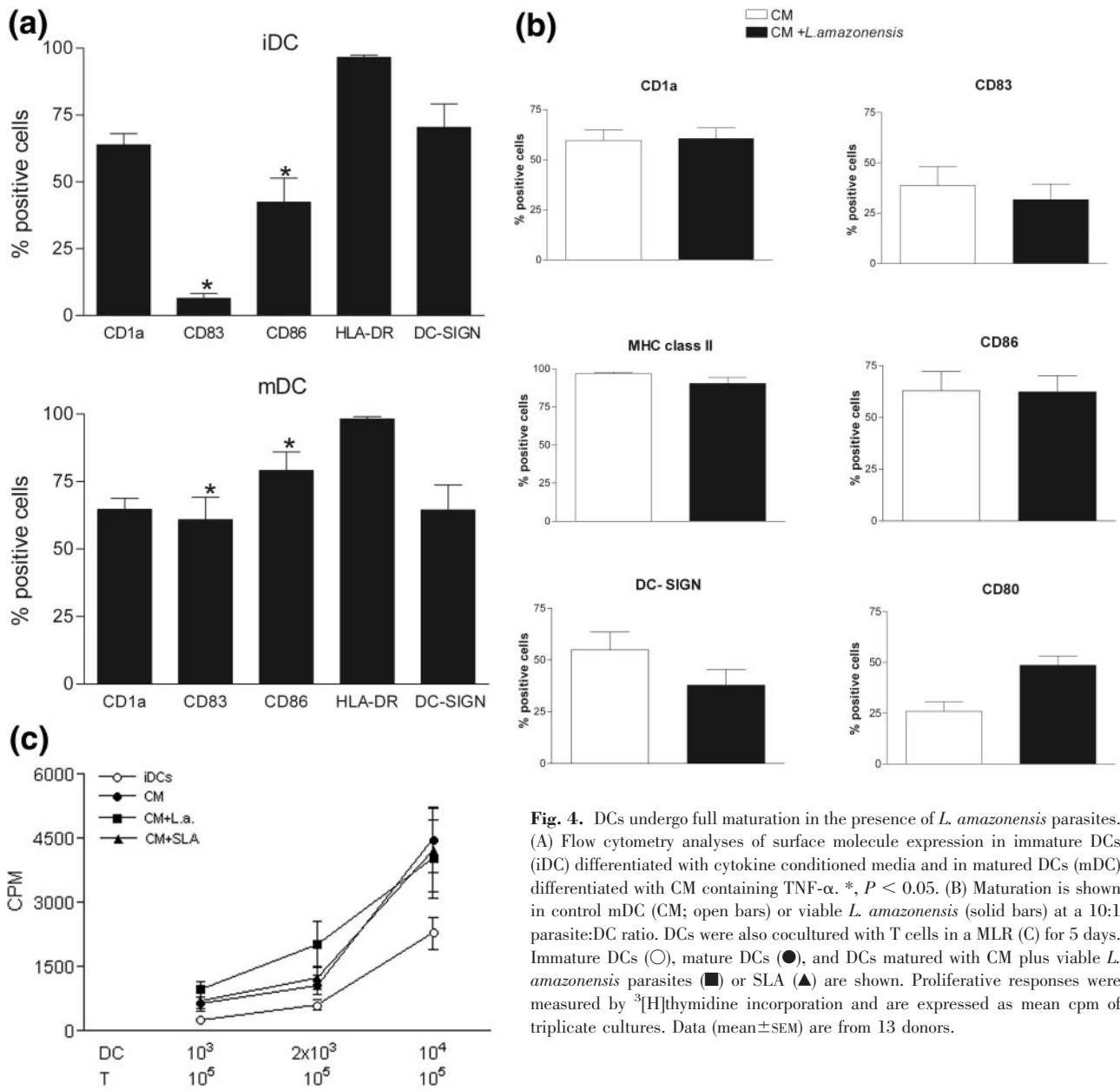
We verified that *L. amazonensis* is able to infect human monocyte-derived DCs and that parasites can interfere with DC differentiation. Our data showed a homogenous rate of DC infection and parasite burden, minimizing the individual variations generally observed with human donors. In this way, our results during differentiation of monocyte-derived DCs showed that whole, viable or heat-killed *L. amazonensis* parasites affected the expression of CD80, CD86, and CD1a. Probably, the parasite products responsible for such incomplete differentiation are structural ones, rather than metabolic, and possibly include those that are lost in SLA preparation, such as glycolipids or lipids [12]. It is important to note that the presence of parasite during differentiation led to a significantly lower expression of CD1a, a DC marker, and a higher expression of CD86. In this way, cells obtained in the parasite's presence cannot be phenotypically defined as DCs; they can be more likely characterized as macrophages, the main host cell for *Leishmania*. Prina et al. [13] observed a similar effect in murine DCs interacting with *L. amazonensis* parasites: an up-regulation of CD86 in infected and uninfected DCs, resulting in "trans-activation" of uninfected cells, probably through the stimulation of the infected ones. Another possibility is the communication through nanotubules in cultured DCs [14]. This also results in a less-intense MLR and probably the loss of

APC characteristics, showing that *L. amazonensis* modifies the DC development pathway. This is especially interesting in an inflammatory context, where blood monocytes are a good source of inflammatory DCs, responsible for an adequate immune response against the parasite [15].

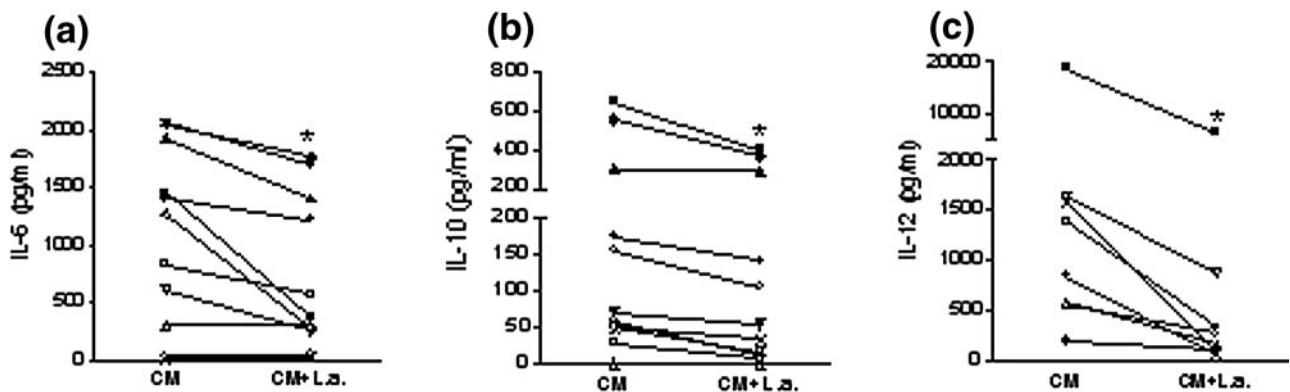
Data from the literature showed differences in the gene expression profiles of *Leishmania*-infected DCs. These studies showed that DCs infected with *Leishmania major* and *Leishmania donovani* had an increased expression of genes associated with inflammatory responses, but *L. major* were more effective in inducing such inflammatory responses [16]. *L. donovani* infection inhibits CD1a, -b, and -c expression in human DCs, with an expressive gene down-regulation, differing from DC incubation with latex beads or heat-killed parasites. Infected DCs were unable to stimulate T cell proliferation and exhibited significant decreased IFN- $\gamma$  production, suggesting that *L. donovani* modulates infected, immature DCs as a pathway for immune evasion, with important implications in visceral leishmaniasis pathogenesis [17]. We could not detect IL-10 and IL-12 in culture supernatants during differentiation, but TNF- $\alpha$  and IL-6 were present. Although the role of TNF- $\alpha$  in inhibiting monocyte-to-DC differentiation has already been described [18], this cytokine seems not to exert these effects in our system. However, we observed a significant decrease in IL-6 production in DC culture supernatants during DC differentiation from monocytes in the presence of *L. amazonensis*. IL-6 is involved in the switching of monocytes from DC to macrophages [19]. C-reactive protein (CRP) substantially down-regulated expression of costimulatory molecules when added during differentiation of DC from human monocytes. CRP-treated DC decreased production of proinflammatory and inflammatory cytokines such as IL-6 [20]. Further experiments are needed to make clear the role of IL-6 in the differentiation of DCs from human monocytes in the presence of *L. amazonensis*. In vitro experiments showed that exogenous IL-6 added to human monocyte cultures impaired the DC differentiation, concluding that IL-6 exerted inhibitory effects on DC generation from monocytes by inducing monocytes to become macrophages [21].

The effects observed in this study about DC biology were also described for other pathogens, such as *B. malayi* microfilaria [4], *Candida albicans* [22], and *P. falciparum* [23]. There is interference in DC differentiation from blood precursors; in this way, T cell immunity is compromised, favoring pathogen survival. Our results showed that *L. amazonensis* altered DC differentiation, causing these cells to become less effective in inducing adaptive immune responses against the parasite.

We observed a lower production of IFN- $\gamma$  in the supernatants of autologous cultures when DCs, differentiated in the presence of parasites, were used as APC. This ineffective, adaptive immune response could be essential for the survival of *L. amazonensis* in infected host cells. This fact is important, as recent data in the literature showed in a mouse model that dermal monocyte-derived DCs are essential in the induction of the protective Th1 response against *Leishmania* in vivo. Dermal monocyte-derived DCs are responsible for the induction of protective immune responses against *L. major*, and especially, inflammatory monocyte-derived DCs are essential in T cell responses against the pathogen. In the early phases of infec-



**Fig. 4.** DCs undergo full maturation in the presence of *L. amazonensis* parasites. (A) Flow cytometry analyses of surface molecule expression in immature DCs (iDC) differentiated with cytokine conditioned media and in matured DCs (mDC) differentiated with CM containing TNF- $\alpha$ . \*,  $P < 0.05$ . (B) Maturation is shown in control mDC (CM; open bars) or viable *L. amazonensis* (solid bars) at a 10:1 parasite:DC ratio. DCs were also cocultured with T cells in a MLR (C) for 5 days. Immature DCs (○), mature DCs (●), and DCs matured with CM plus viable *L. amazonensis* parasites (■) or SLA (▲) are shown. Proliferative responses were measured by  $^3\text{H}$ thymidine incorporation and are expressed as mean cpm of triplicate cultures. Data (mean  $\pm$  SEM) are from 13 donors.



**Fig. 5.** *L. amazonensis* down-modulates cytokine production during DC maturation. Cytokine detected on culture supernatants measured after 48 h culture of DCs matured with CM (TNF- $\alpha$ ) only or in the presence of *L. amazonensis* parasites (a) IL-6, (b) IL-10, and (c) IL-12. Each point represents one donor. \*,  $P < 0.05$ .

tion, free parasites could gain access to draining lymph nodes and infect resident DCs. At later stages, amastigotes, released in the dermis from previously infected cells, would infect dermal monocyte-derived DCs, which would then be responsible for carrying the infection to the lymph nodes and inducing the activation of *Leishmania*-specific Th1 responses [24]. This mechanism was shown in the mouse model but probably can occur in human infection by *Leishmania*, and our results show that the presence of parasites interferes with the differentiation of DCs from monocytes, contributing to the persistence of the parasite. It is important to point out that *L. amazonensis* is responsible for causing DCL, a rare form of cutaneous leishmaniasis, where patients show nodules with parasites and a lack of IFN- $\gamma$  production. We can speculate that this effect of *L. amazonensis* on monocyte-derived DC differentiation observed in our experiments could be one of the factors, which contributes to this severe form of disease. The presence of *L. amazonensis* or SLA during maturation of DCs resulted in no differences on cell surface molecule expression or MLR intensity when these cells were used as stimulators. Of note, pathogens such as *P. falciparum* [3] or *Mycobacterium leprae* [25] are able to abrogate DC maturation. Despite the lack of significant differences on cell surface molecule expression, we observed decreased levels of IL-6, IL-10, and IL-12 when maturation stimuli were added simultaneously to *L. amazonensis* parasites compared with control DCs. This suggests that the parasite down-regulates different signaling pathways, precluding the appropriate activation of cellular immune responses. Kranzer et al. [26] showed that incubation of DCs with viable or formalin-inactivated *Helicobacter pylori* also induced comparable levels of IL-6, IL-8, IL-10, IL-12, IL-1 $\beta$ , and TNF- $\alpha$  but a reduction in IL-12 and IL-1 $\beta$  secretion. This effect was related to phagocytosis of *H. pylori* [27].

We showed that *L. amazonensis* parasites manifest the ability to alter DC differentiation from human monocytes, causing a down-regulation of the Th1-adaptative immune response, and thus, favoring its survival in infected human host cells.

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