Visceral leishmaniasis – malaria co-infections

Epidemiological, immunological and parasitological aspects

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Leishmania donovani infection drives the priming of human monocyte-derived dendritic cells during Plasmodium falciparum co-infections

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Summary

Functional impairment of dendritic cells (DCs) is part of a survival strategy evolved by *Leishmania* and *Plasmodium* parasites to evade host immune responses. Here, the effects of co-exposing human monocyte-derived DCs to *Leishmania donovani* promastigotes and *Plasmodium falciparum*-infected erythrocytes were investigated. Co-stimulation resulted in a dual, dose-dependent effect on DC differentiation which ranged from semi-mature cells, secreting low interleukin-12p70 levels to a complete lack of phenotypic maturation in the presence of high parasite amounts. The effect was mainly triggered by the *Leishmania* parasites, as illustrated by their ability to induce semi-mature, interleukin-10-producing DCs, that poorly responded to lipopolysaccharide stimulation. Conversely, *P. falciparum* blood-stage forms failed to activate DCs and only slightly interfered with lipopolysaccharide effects. Stimulation with high *L. donovani* concentrations triggered phosphatidylserine translocation, whose onset presented after initiating the maturation impairment process. When added in combination, the two parasites could co-localize in the same DCs, confirming that the leading effects of *Leishmania* over *Plasmodium* may not be due to mutual exclusion. Altogether, these results suggest that in the presence of visceral leishmaniasis-malaria co-infections, *Leishmania*-driven effects may overrule the more silent response elicited by *P. falciparum*, shaping host immunity towards a regulatory pattern and possibly delaying disease resolution.

**Keywords**

Co-infection, Dendritic cell, *L. donovani*, *P. falciparum*
Introduction

Malaria and visceral leishmaniasis (VL) are two major parasitic diseases sharing part of their geographical distribution across the globe (Figure 1).\textsuperscript{1,2} Transmitted through the bite of infected vectors with similar feeding habits and exacerbated by the lack of protective immunity found in children, the two diseases can co-occur in individuals living in co-endemic areas, with potential implications for their clinical course.\textsuperscript{3-7} Recent studies conducted in East Africa, where the co-infection prevalence in patients with VL ranged from 4% to 61%, highlighted a substantial increase in disease-related morbidity, with co-infected patients suffering from more frequent emaciation, jaundice and malaise, despite exhibiting a similar prognosis.\textsuperscript{6,7} Interestingly, VL and malaria have been shown to cross-interact in experimentally co-infected animals, shaping host susceptibility towards one or the other infection and the immune response elicited locally, albeit with substantial differences across the various studies.\textsuperscript{8-11} At the patient level, increased concentrations of Th1 and pro-inflammatory cytokines were observed amongst Sudanese co-infected individuals, demonstrating the ability of the two pathogens to modulate host immunity and possibly the severity of infections that follow.\textsuperscript{12}

Crucial to the immune evasion strategies evolved by several pathogens is their ability to interfere with the function of dendritic cells (DCs), a group of professional antigen-presenting cells that coordinate innate and adaptive immunities.\textsuperscript{13,14} The result is an impairment of DC activities which manifests with host inability to mount a full adaptive immune response and clear infection. Resistance to intracellular pathogens, such as Leishmania, requires induction of immune responses capable of activating cellular microbicidal mechanisms. To this end, priming of DCs to release interleukin(IL)-12 is pivotal for promoting Th1 differentiation and the production of interferon-gamma (IFN-γ), the most potent cytokine for the induction of leishmanicidal activity in macrophages. Interestingly, despite the early finding that some IL-12 may be pre-stored and rapidly demobilized following Leishmania contact,\textsuperscript{15,16} in vitro studies have collectively indicated that DCs exposed to L. donovani promastigotes fail to mature in response to parasite engulfment and produce a range of IL-12p40 and IL-10 concentrations.\textsuperscript{17,18} Likewise, reduced responsiveness of L. donovani-infected DCs was observed following addition of exogenous stimuli, such as lipopolysaccharide (LPS)/tumor necrosis factor-alpha (TNF-α), IFN-γ, CD40 ligand or Mycobacterium tuberculosis,\textsuperscript{17-20} suggesting that Leishmania promastigotes may not only act by evading engagement of pathogen-recognition receptors (PRRs) associated with DC activation and T cell priming, but also by actively suppressing DC signaling pathways. Importantly, these findings appear to be model- and stage-specific, as evidence obtained in vivo or using the amastigote stage indicates up-regulation of DC maturation-associated cell-surface markers, release of cytokines and induction of Th1 responses in naïve CD4\textsuperscript{+} T cells.\textsuperscript{18,21} In further contrast, Leishmania dermatotropic species have been shown to produce a variety of effects on the biology and functions of DCs, ranging from a simple delay in DC maturation, as the one promoted by L. major,\textsuperscript{22} to a severe inhibition of DC signaling pathways by L. amazonensis and L. mexicana.\textsuperscript{23-25}

Impairment of DC phenotype and activities is part of a pathogen survival strategy exploited by the Plasmodium malaria parasite for improving
its fitness.\textsuperscript{26,27} Consistent with its two phase-cycle (hepatic and intra-erythrocytic) in the mammalian host, control of \textit{Plasmodium} infection by the host requires a multifaceted immune response, consisting of cytotoxic CD8\(^+\) T cells to suppress the liver forms and CD4\(^+\) T lymphocytes to clear blood parasites, first via cell-mediated immunity stimulated by Th1 differentiation and ultimately by antibody opsonization.\textsuperscript{27} DC activation of CD8\(^+\) T cells seems to be unaffected by the pre-erythrocytic stage of infection,\textsuperscript{28-32} whereas blood-stage forms are known to deeply modulate the functional capacity of various DC subsets. Several \textit{in vitro} and \textit{in vivo} studies conducted with human myeloid DCs have shown reduced DC activity following exposure to erythrocytes infected with \textit{P. falciparum} or hemozoin, its by-product of hemoglobin degradation.\textsuperscript{33-37} The effect appears to be dose-dependent, with low parasite doses resulting in DC activation and high doses causing DC suppression via apoptosis induction.\textsuperscript{34} Analysis of DC function in rodent \textit{Plasmodium} infections, however, has only partially confirmed these results, providing evidence that the functional capacity of splenic CD11c\(^+\) DC populations changes over the course of infection and the lethality of the infecting strain.\textsuperscript{34,38-40} Whilst early on in infection when parasite density is still low, IL-12-mediated mechanisms induce IFN-\(\gamma\)-producing CD4\(^+\) T cells, the phenotype of DCs may change as the infection progresses.\textsuperscript{39,41} With lethal strains, in particular, DCs may become refractory to Toll-Like Receptors (TLR) and other signaling events, inhibiting IL-12 and TNF-\(\alpha\) secretion and promoting induction of regulatory IL-10-secreting T cells, deleterious for parasite clearance.\textsuperscript{40,42}

In the present study, the effect of \textit{in vitro} concomitant exposure to \textit{L. donovani} promastigotes and \textit{P. falciparum}-infected erythrocytes on DC function was examined. Expression of co-stimulatory molecules along with cytokine release was measured after co-stimulating monocyte-derived DCs (mo-DCs) with increasing amounts of parasite, in the presence or absence

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**Figure 1.** Overlap in the geographical distribution of visceral leishmaniasis (reported cases) and malaria cases (reported confirmed cases, microscopy slides/RDTs positive), as obtained by combining data reporting the distribution at country-level of the two diseases individually in 2012 (Global Health Observatory Data Repository. 2008. Geneva: World Health Organization; <http://apps.who.int/ghodata/> [accessed June 2014]).
of an exogenous stimulus (LPS). Cellular and transcriptional expression of various PRRs (DC-SIGN, TLR2 and TLR4) was assessed for each of the examined stimuli, whose effect on apoptosis was also examined through assessment of phosphatidylserine (PS) externalization.

Materials and methods

Isolation of human monocytes and DC generation

Human monocytes derived from healthy donors (Sanquin, Amsterdam, the Netherlands; Approval Nr. NVT0224.01-04) who had signed informed consent for research purposes, were isolated from fresh buffy coats, by centrifugation over a gradient of Ficoll-Paque Plus, followed by 46% iso-osmotic Percoll (both from GE Healthcare, Uppsala, Sweden). After isolation, monocytes were cultured at a final concentration of 1.5 X 10^6 cells/mL in RPMI-buffered medium (RPMI 1640 medium containing 25 mM HEPES and 2 mM L-glutamine) (Gibco, Bleiswijk, the Netherlands), supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich, St Louis, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin. Differentiation into immature mo-DCs was achieved in the presence of 20 ng/mL of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (both from Life Technologies, Bleiswijk, the Netherlands), which were added to the above-mentioned culture medium (DC culture medium). After 4 or 5 day-differentiation, immature mo-DCs were transferred to 96-well round-bottom culture plates (1X10^6 cells/well) and stimulated for 24-h (unless otherwise stated) with increasing doses of L. donovani 1S promastigotes, uninfected red blood cells (RBCs), P. falciparum-infected RBCs or a combination of L. donovani 1S promastigotes and P. falciparum-infected RBCs, followed by 48-h in the presence or absence of LPS (from E. coli 0111:B4, Sigma-Aldrich Co.). The amounts of parasite used for mo-DC (co)-stimulation were determined based on previous studies exploring the effect of malaria and VL mono-infections on DC function.17,19,34,43,44

Cultivation of P. falciparum-infected RBCs and enrichment of late-stage parasite cultures

Parasites of the P. falciparum strain 3D7 (MR4/ATCC: MRA-102) were grown at 5% hematocrit (0% human erythrocytes) in RPMI-buffered medium supplemented with 10% heat-inactivated human AB+ serum (Sanquin) and 50 μg/mL gentamycin. Cultures were maintained at 37°C in candle jars, as described by Trager and Jensen,45 under continuous agitation at 40 rpm/min and with daily refreshment of medium. When parasitaemia exceeded 7-8%, sub-cultures were made.

For stimulation of immature mo-DCs, highly enriched, late-stage P. falciparum cultures were prepared according to Wahlgren et al.,46 with minor modifications. Briefly, mature-stage parasite cultures were washed three times with RPMI-buffered medium and fractionated at 10% hematocrit onto a 60% Percoll gradient. After centrifugation at 1500 g, 4°C for 15 min, late-stage P. falciparum-infected RBCs (trophozoite and schizont forms) were harvested from the top of the Percoll solution, while uninfected and ring-containing RBCs pelleted at its bottom. Infected RBCs were washed three times with phosphate-buffer saline pH 7.2 (PBS) to remove residual Percoll and re-suspended in DC culture medium at a final concentration of 2.5 X 10^8 RBCs/mL. The enriched fractions had a mean parasitaemia purity of 75%, as assessed by microscopic examination of Field’-stained smears. As controls, uninfected RBCs were cultured for 3 to 4 days in the same conditions and similarly processed onto
the 60% Percoll solution, from which they were retrieved at the lowest layer. The uninfected RBCs were then washed and re-suspended as for the enriched \textit{P. falciparum}-infected cells.

\textbf{Cultivation of \textit{L. donovani} promastigotes}

Promastigotes of the Sudanese \textit{L. donovani} strain 1S (MHOM/SD/1968/1S) were cultured as previously reported,\textsuperscript{47} with minor modifications. Briefly, promastigotes were maintained at 27°C with a weekly passage in RPMI-buffered medium supplemented with 15% heat-inactivated FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin.

Parasites used for cell stimulation were harvested from 5-day-old cultures (stationary phase) and re-suspended in DC culture medium at a final concentration of $2.5 \times 10^8$ parasites/mL.

\textbf{Flow cytometric analysis}

Fluorescence-activated cell sorting (FACS) analysis was used to analyze cell-surface expression of DC marker CD11c, maturation markers CD80, CD86, CD40 and HLA-DR, the adhesion molecule CD209 (DC-SIGN), and the PRRs CD282 (TLR-2) and CD284 (TLR-4). Prior to immunostaining, cells were treated with an RBC lysing buffer to remove non-internalized RBCs, else interfering with the FACS analysis, and incubated for 30 min at 4°C with a mixture of the following fluorescent-labeled monoclonal anti-human antibodies (all from BioLegend, San Diego, USA), according to the manufacturer’s instructions: fluorescein isothiocyanate (FITC)-anti-CD11c, phycoerythrin (PE)-anti-CD80, Pacific Blue-anti-CD86, allophycocyanin (APC)-anti-CD40, allophycocyanin-cyanine 7 (APC-Cy7)-anti-HLA-DR, peridin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5)-anti-CD209, Alexa Fluor 647-anti-CD282 and PE-anti-CD284. Thereafter, cells were fixed with 2% paraformaldehyde in PBS, re-suspended in FACS buffer (PBS pH 7.2 supplemented with 0.5% bovine serum albumin and 0.02% azide) and acquired on an LSR Fortessa (BD Biosciences, San Jose, USA). A minimum of 10,000 events was measured per sample. A gate based on forward and side scatters was set to exclude cell debris and non-internalized parasites. The mean fluorescence of gated cells was determined, and the results were analyzed using the FlowJo software (Tree Star Inc., Ashland, USA). Annexin V staining was used to assess cell-surface expression of PS, a phospholipid normally confined to the inner leaflet of the plasma membrane, which serves as a ligand for receptor-mediated phagocytosis. Although not limited to,\textsuperscript{48} cells undergoing apoptotic cell death typically externalize PS, whose detection serves as apoptotic marker. Briefly, cells were treated as described above, incubated for 30 min at 4°C with FITC-labeled annexin V (Bio-Legend) and after fixation, re-suspended in binding buffer (10 mM HEPES, 140 mM NaCl, 25 mM CaCl\textsubscript{2}). Monitoring of necrotic cells was not performed due to incompatibility between the propidium iodide assay and cell fixation, required for parasite inactivation prior to FACS analysis.

\textbf{Cytokine measurements}

For detection of cytokines, DC culture supernatants were harvested at different time points after stimulation and frozen at -20°C until analysis. Levels of IL-10 and IL-12p70 were measured by enzyme-linked immunosorbant assay (ELISA) using Legend Max\textsuperscript{™} ELISA kits (BioLegend), performed according to manufacturer’s instructions. Detection of human TNF-α was performed by ELISA using an antibody sandwich pair (Invitrogen, Bleiswijk, the Netherlands). Plates were first coated overnight at 4°C with the capture antibody diluted in 0.05 M Na\textsubscript{2}CO\textsubscript{3}. Thereafter, plates were washed in PBS/0.02%
DC maturation during VL-malaria co-infections

Tween® 20 (Merck KGaA, Darmstadt, Germany) and blocked with PBS/1% bovine serum albumin fraction V (Roche Diagnostics, Basel, Switzerland), prior to the addition of the sample together with the detection antibody. The reaction was monitored at 450 nm with an Infinite M200Pro multimode plate reader (Tecan, Männendorf, Switzerland), after halting the oxidation of 3,3’5,5’-tetramethylbenzidine (TMB) catalyzed by streptavidin-horseradish peroxidase with 0.8 M sulfuric acid (H$_2$SO$_4$).

**mRNA isolation and complementary DNA (cDNA) synthesis**

Real-time quantitative Reverse Transcriptase-PCR (qRT-PCR) was performed to assess mRNA expression of TLR2, TLR4 and DC-SIGN. Total DNA and RNA was isolated from (un)stimulated DCs, as described by Boom et al., and DNase-digested using the Turbo DNA-free kit (Ambion, Austin, USA), according to the manufacturer’s procedures. To confirm the complete removal of genomic DNA, qPCR targeting two of the five target genes was performed after each DNase treatment on a randomly selected set of samples. Prior to real-time quantification, 20 µL of cDNA synthesis mix consisting of 0.03 µg/µL random hexamers primers (Roche Diagnostics), 5 mM MgCl$_2$, 0.48 mM dNTP (Thermo Scientific, Waltham, USA), 0.08 U/µL RNasinPlus RNase inhibitor (Promega, Madison, USA), 0.4 U/µL SuperScript II reverse transcriptase (Life Technologies) in 1X CBM buffer was mixed with 80 µL of each DNA-free RNA sample, and incubated at 42°C for 30 min or longer to enable synthesis of cDNA from the RNA templates. Samples were subsequently stored at -70°C until further analysis.

**qRT-PCR**

Amplification of TLR2, TLR4 and DC-SIGN sequences was performed in separate reactions, each of which included the household single copy gene β-2-microglobulin (β-2M) to correct for exact cell number and to control for test performance. Primers (Eurogentec, Maastricht, the Netherlands) targeting TLR2, TLR4 and DC-SIGN mRNA were designed to amplify intra-exon sequences, while intron-spanning primers were used to selectively amplify β-2M cDNA over its genomic counterpart, providing an internal control for cDNA synthesis (Table 1). Detection of DNA amplification was performed by sequence-specific probes (Eurogentec) labeled with the fluorophore FAM (Table 1). The qPCR reaction mix for TLR2, TLR4 and β-2M sequences (final volume 25 µL) consisted of 2.5 µL of 10X PCR buffer (Qiagen, Venlo, the Netherlands), 200 nM forward primer, 200 nM reverse primer, 40 nM probe, 0.5 U Hotstart Taq polymerase (Qiagen), 400 nM dNTP, 18.3 µL water and 2.5 µL cDNA. For DC-SIGN, the qPCR reaction mix was similarly composed, except that it contained 2 mM MgCl$_2$ (Qiagen) and 17.8 µL water. The qPCR was performed in a Biorad CFX96 real-time detection system (Bio-Rad, Hercules, USA) using the following cycling conditions: 10 min at 95°C, followed by 40 cycles, each consisting of 20 s at 95°C and 1 min at 60°C, during which the emitted fluorescence is measured. In each qPCR experiment, a negative control (water), an extraction control and a 5-step dilution series of parasite or cell cDNA were simultaneously assessed to ensure for high-quality DNA extraction and good performance/efficiency of the PCR. Percentage expression of target genes TLR2, TLR4 and DC-SIGN was calculated against unstimulated and LPS-treated cells, respectively, after computing a relative expression ratio against the reference gene β-2M on the basis of
the PCR efficiency and crossing point deviation of the investigated transcripts.\textsuperscript{51}

**Statistical analysis**

Expression of co-stimulatory molecules and DC-SIGN in response to *L. donovani* and/or *P. falciparum* stimulation was measured on mo-DCs obtained from five different donors, three of whom were examined for cytokine release, too. Statistical analysis and data shown in the present manuscript refer to these three donors for whom phenotypic data were matched by cytokine release activity (a substantially similar trend in phenotype was evident with mo-DCs from these 3 as well as from all 5 donors).

Statistical analyses were performed with the software package GraphPad Prism (Software Inc., San Diego, USA). For paired comparisons of two groups, a paired sample *t*-test was performed. For other parametric data, a one-way analysis of variance (ANOVA) was performed, followed by *post hoc* Dunnett’s multiple comparison test. A *P* value <0.05 was considered statistically significant. *P* < 0.05, **P** < 0.01, ***P*** < 0.001 indicate values that are significantly different from the unstimulated DCs. *P* < 0.05, **P** < 0.01, and ***P*** < 0.001 indicate values that are significantly different from the LPS-stimulated DCs.

### Results

*L. donovani* promastigotes promote semi-mature, anergic IL-10-producing mo-DCs that interfere with LPS-triggered activation.

To investigate the effect of VL on DC function, the activation status of mo-DCs obtained from three different donors and exposed to various amounts of *L. donovani* 1S promastigotes was examined through their phenotype and cytokine patterns. Immature mo-DCs responded to stimulation with Leishmania parasites in a dual fashion (Figure 2a, S1). At lower parasite amounts (mainly 1 to 10 promastigotes per mo-DC), mo-DCs up-regulated co-stimulatory molecules CD80 (*P* < 0.05 with *Ld* 5) and CD86 (*P* < 0.05 with *Ld* 10 and *Ld* 25), with a little decrease in CD40 and HLA-DR cell levels. This phenotype interposed between the one of immature and LPS-activated cells, indicating that DCs stimulated with low amounts of *L. donovani* promastigotes only reached a semi-mature state. At higher parasite-to-cell ratios, conversely, a decrease for most of the examined maturation markers was observed (*P* < 0.05 for CD80, *P* < 0.01 for CD40 with *Ld* 25, *P* < 0.05 for *Ld* 10 and *Ld* 25), with a little decrease in CD40 and HLA-DR cell levels. This phenotype interposed between the one of immature and LPS-activated cells, indicating that DCs stimulated with low amounts of *L. donovani* promastigotes only reached a semi-mature state. At higher parasite-to-cell ratios, conversely, a decrease for most of the examined maturation markers was observed (*P* < 0.05 for CD80 and CD40 with *Ld* 25, *P* < 0.01 for CD40 with *Ld* 50 and *P* < 0.001 for CD80 with *Ld* 50), suggesting either a general parasite-induced toxicity or an active inhibition of DC functions. Cytokine

\textbf{Table 1. Nucleotide sequences of the PCR primers and probes used to assay gene expression by qRT-PCR.}

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
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<tbody>
<tr>
<td>TLR2</td>
<td>5’ GGCCAGCAAAT TACCTGTTG 3’</td>
<td>5’ GAGCAGGATCA GCAGGAACAC 3’</td>
<td>5’ FAM-CTCTCTCGGT GTCCGAAGTG-BHQ1 3’</td>
</tr>
<tr>
<td>TLR4</td>
<td>5’ CAGAGCCCGCTG GTGTATC 3’</td>
<td>5’ TCCCACCTCCAG GTAAGTTGGT 3’</td>
<td>5’ FAM-TGAATATGAGA TGGTCAGACCT-BHQ1 3’</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>5’ GTCCCTCAGTG GACGAAGTT 3’</td>
<td>5’ GCCCTGAAGAA GGAGAAAGGA 3’</td>
<td>5’ FAM-CTTGCTGGCG TTTCT-BHQ1 3’</td>
</tr>
<tr>
<td>β-2-Microglobulin</td>
<td>5’ GGCTATCCAGC GTACTCAA 3’</td>
<td>5’ GATGAAAACCAC GACACATAGCA 3’</td>
<td>5’ FAM-ATCTACGTCA TCCAGCAGAG-BHQ1 3’</td>
</tr>
</tbody>
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release (Figure 3a) was characterized by up-regulation of IL-10 production and a substantial, although statistically not significant, decrease in IL-12p70 levels. These changes were greatest in immature mo-DCs stimulated with equal amounts of *Leishmania* parasites (parasite-to-cell ratio of 1:1). Secretion of TNF-α followed a dose-dependent increase up to 10 parasites per mo-DC, but the raise lacked statistical power.

When mo-DCs pre-incubated with *L. donovani* were stimulated with LPS, only the lowest parasite doses (parasite-to-cell ratios of 1:1 and 5:1 partially) displayed a slight additional effect on LPS-induced expression of co-stimulatory molecules, whereas higher parasite amounts caused the expression of all maturation markers to progressively decrease to nullify the effect of LPS (*P* < 0.05 for CD80 with *Ld* 25, *P* < 0.01 for CD80 and CD40 with *Ld* 50 and *Ld* 25, respectively and *P* < 0.001 for CD40 with *Ld* 50) (Figure 2a, S1). *L. donovani*-mediated interference of LPS effects was also evident on IL-10 and IL-12p70, whose levels in cell supernatants decreased following a dose-dependent fashion (up to 10 parasites per mo-DC for IL-12p70) (Figure 3a).

**P. falciparum** fails to activate mo-DCs and partially interferes with LPS-triggered activation

Immature mo-DCs exposed to allogeneic erythrocytes responded with an overall, minimal increase in the expression of DC maturation markers (Figure 2b, S2a). Similarly, a modest increase in IL-10, TNF-α and particularly IL-12p70 release was observed for almost all concentrations of RBCs (Figure 3b), but no statistically significant difference could be observed. Importantly, uninfected RBCs displayed a stable effect on both phenotype and cytokine profiles, with most changes exhibiting a dose-independent pattern.

When the same allogeneic erythrocytes were infected with *P. falciparum*, exposure to immature mo-DCs (isolated from the same donors as for stimulation with uninfected RBCs and *L. donovani*) barely altered their phenotypic maturation, with the strongest effects being displayed on CD80 (*P* < 0.05 with *PfRBC* 10 and *P* < 0.01 with *PfRBC* 25) and HLA-DR (*P* < 0.05 with *PfRBC* 50) (Figure 2c, S2b). Little effects were observed on cytokine release, too, which registered a modest, dose-dependent increase in IL-10 and TNF-α (starting from *PfRBC* 5) and a more pronounced, but highly variable, enhancement in IL-12p70 (Figure 3c). Comparison of mo-DCs stimulated with the same *P. falciparum*-infected and uninfected erythrocyte populations (isolated from the same donor) demonstrated a partial inhibition of RBC-triggered effects (Figure 2b and 2c, S2a and S2b). Whilst a dose-dependent increase in CD80 and CD86 levels was observed, sufficient to outnumber only RBC-induced CD80 (*P* < 0.01 with *PfRBC* 5), expression of CD40 and HLA-DR progressively diminished as the parasite-to-cell ratio augmented (*P* < 0.05 with *PfRBC* 50) (statistical data not shown).

A similar *P. falciparum*-triggered interference was observed with regard to the LPS-induced activation. Mo-DCs stimulated with uninfected RBCs 24-h prior to LPS displayed similar features to the LPS-exposed cells, both in terms of phenotype (Figure 2b, S2a) and cytokine production (Figure 3b). By contrast, mo-DCs pre-incubated with *P. falciparum*-infected RBCs failed to fully mature in response to LPS stimulation (*P* < 0.05 for CD40 with *PfRBC* 5 and *P* < 0.001 with *PfRBC* 25 and *PfRBC* 50), whose effects were partially inhibited in a dose-dependent fashion (Figure 2c, 3c, S2b).
Figure 2. Expression of surface markers on monocyte-derived dendritic cells (mo-DCs) in response to parasite stimulation. Immature mo-DCs were cultured alone or in the presence of either \textit{L. donovani} promastigotes (a), uninfected RBCs (b), \textit{P. falciparum}-infected RBCs (c) or \textit{L. donovani} promastigotes and \textit{P. falciparum}-infected RBCs (d) at different parasite-to-cell ratios (ranging from 1:1 to 50:1) for 24-h, followed by 48-h of incubation in the presence or absence of LPS (20 ng/mL). Thereafter, cells were harvested, and expression of maturation markers was measured by flow cytometry. Data represent means ± SEMs (error bars) of three independent experiments performed with DCs from different donors and are indicated as the percent fold-change of activity vs. the unstimulated DCs (100%), calculated by pooling the percent fold-change of each individual experiment. \textit{Ld} = \textit{L. donovani}; \textit{PfRBC} = \textit{P. falciparum}-infected RBC; \textit{Ld-PfRBC} = \textit{L. donovani} and \textit{P. falciparum}-infected RBC. *\textit{P} \textless 0.05, **\textit{P} \textless 0.01, ***\textit{P} \textless 0.001 for values that are significantly different from the unstimulated mo-DCs. *\textit{P} \textless 0.05, **\textit{P} \textless 0.01, and ***\textit{P} \textless 0.001 for values that are significantly different from the LPS-stimulated mo-DCs.
Figure 3. Cytokine secretion profiles of mo-DCs in response to parasite stimulation. Immature mo-DCs were cultured alone or in the presence of either *L. donovani* promastigotes (a), uninfected RBCs (b), *P. falciparum*-infected RBCs (c) or *L. donovani* promastigotes and *P. falciparum*-infected RBCs (d) at different parasite-to-cell ratios (ranging from 1:1 to 50:1) for 24-h, followed by 48-h of incubation in the presence or absence of LPS (20 ng/mL). Thereafter, cell supernatants were collected and levels of IL-10, IL-12p70 and TNF-α were measured by ELISAs. Data represent means ± SEMs (error bars) of three independent experiments performed with DCs from different donors and are indicated as the percent fold-change of activity vs. the unstimulated DCs (100%), calculated by pooling the percent fold-change of each individual experiment. Cytokine control levels of the three donors varied between 14.6 and 195.8 pg/mL for IL-10 (mean 100% value = 78.3 pg/mL), 2.6 and 51.5 pg/mL for IL-12p70 (mean 100% value = 18.5 pg/mL), 262.7 and 10062.0 pg/mL for TNF-α (mean 100% value = 4672.6 pg/mL). *Ld* = *L. donovani*; *Pf RBC* = *P. falciparum*-infected RBC; *Ld-Pf RBC* = *L. donovani* and *P. falciparum*-infected RBC. No statistically significant difference was found.
Leishmania drives the priming of mo-DCs co-exposed to L. donovani promastigotes and P. falciparum-infected RBCs

Co-exposure of mo-DCs (isolated from the same donors as for the above stimulation) to L. donovani promastigotes and P. falciparum-infected RBCs resulted in a phenotype largely mirroring the Leishmania-driven scenario (Figure 2d, S3). Whilst low doses of parasites triggered a semi-maturation state, marked by a significant up-regulation of CD80 ($P < 0.05$ with Ld-PfRBC 5) and CD86 levels ($P < 0.05$ with Ld-PfRBC 10), higher amounts of parasite significantly decreased the expression of CD80 ($P < 0.05$ with Ld-PfRBC 25 and $P < 0.01$ with Ld-PfRBC 50) and CD40 ($P < 0.05$ with Ld-PfRBC 10, $P < 0.01$ with Ld-PfRBC 25 and 50). This dual, dose-dependent effect was evident in the cytokine release, too, which peaked at parasite-to-cell ratios of 10:1 for IL-10 and TNF-α, and of 5:1 for IL-12p70, while reaching its minimum with the highest parasite dose (Figure 3d). None of the examined stimuli, however, was sufficient to significantly induce or inhibit secretion of the three cytokines.

Cell response to LPS stimulation 24 hour after parasite exposure consisted in an unaltered or slightly increased expression of maturation markers at the lowest doses (parasite-to-cell ratios of 1:1 and 5:1 for CD80 and CD86 only), followed by a progressive, dose-related inhibition of phenotypic maturation ($P < 0.05$ for CD86 with Ld-PfRBC 5 and for CD40 with Ld-PfRBC 25 and 50, $P < 0.01$ for CD80 with Ld-PfRBC 25 and 50) (Figure 2d, S3). Similarly, pre-stimulation of mo-DCs with the two parasites prior to LPS negatively affected their IL-10 and TNF-α production in a dose-dependent fashion (Figure 3d).

L. donovani and P. falciparum co-localize in mo-DCs and modulate expression of cellular receptor DC-SIGN

Immature mo-DCs have been shown to successfully phagocytose antigens from the surrounding environment, including parasites of the Leishmania and Plasmodium spp.\textsuperscript{16,52,53} This was evident in our experiments, too (data not shown). In addition, mo-DCs co-exposed to L. donovani promastigotes and P. falciparum-infected RBCs were shown to be potentially permissive towards both parasites, being able to internalize and co-harbor Leishmania as well as Plasmodium late-stage forms (trophozoites and schizonts) (Figure 4).

To investigate whether the driving effect displayed by L. donovani on DC differentiation may have resulted from the inability of Plasmodium parasites to engage its effector receptors, expression levels of DC-SIGN, TLR2 and TLR4 were examined. Mo-DCs (isolated from the three donors as for the previous experiments) exposed to increasing doses of L. donovani promastigotes progressively reduced the amount of C-type lectin DC-SIGN expressed on their surface (Figure 5a, S4). The decrease was evident at low parasite amounts already ($P < 0.05$ with Ld 5 and 10), but it reached high statistical significance power ($P < 0.001$) only at the highest parasite-to-cell ratios (25:1 and 50:1). A similar effect ($P < 0.05$ with Ld 5, $P < 0.001$ with Ld 10, 25 and 50) was observed in the presence of L. donovani and P. falciparum-infected erythrocytes, as confirmed by the comparable levels of DC-SIGN on mo-DCs co-exposed to the two parasites, which reflected the poor ability of uninfected and P. falciparum-infected RBCs to modulate DC-SIGN surface levels (Figure 5a, S4). LPS-induced maturation of mo-DCs significantly reduced the overall expression of DC-SIGN ($P < 0.05$), as to be expected, but
DC maturation during VL-malaria co-infections

Figure 4. Co-localization of *L. donovani* and *P. falciparum* in mo-DCs. Mo-DCs were co-cultured with *L. donovani* promastigotes and *P. falciparum*-infected RBCs. After 12-h of incubation, cells were harvested and optical microscope images (Field'-stained, viewed with a 100X objective) were taken. Several hemozoin crystals (some of which are indicated by the arrows) are visible in the cell, where they persist as remnants of late-stage malaria parasites, along with several *Leishmania* parasites (some of which are indicated by the asterisks), recognizable through their nucleus (pink colored-mass) and kinetoplast (deeply stained, purple organelle).

Figure 5. Expression of DC-SIGN and TLR2 on mo-DCs in response to parasite stimulation. Immature mo-DCs were cultured alone or in the presence of either *L. donovani* promastigotes, uninfected RBCs, *P. falciparum*-infected RBCs or *L. donovani* promastigotes and *P. falciparum*-infected RBCs at different parasite-to-cell ratios (ranging from 1:1 to 50:1) for 24-h, followed by 48-h of incubation in the presence or absence of LPS (20 ng/mL). Thereafter, cells were harvested, and expression of DC-SIGN (a) or TLR2 (b) was measured by flow cytometry. Data represent means + SEMs (error bars) of three independent experiments performed with mo-DCs from different donors and are indicated as the percent fold-change of activity vs. the unstimulated mo-DCs (100%), calculated by pooling the percent fold-change of each individual experiment. *Ld = L. donovani; PfRBC = P. falciparum*-infected RBC; Ld-PfRBC = *L. donovani and P. falciparum*-infected RBC. *P <0.05 and ***P <0.001 for values that are significantly different from the unstimulated mo-DCs. *P <0.05 and ##P <0.01 for values that are significantly different from the LPS-stimulated mo-DCs.
the progressive decrease caused by *L. donovani* was still clearly visible (*P* < 0.01 with *Ld* 25 and 50 and with *Ld*-PfRBC 25 and 50) (Figure 5a, S4). Transcriptional profiling of DC-SIGN (Table S1) showed its diminished detection on cellular surface could not be attributed to a reduction in gene expression, corroborating the prospect of a rapid DC-SIGN complex internalization upon *Leishmania* binding.

Cellular expression of TLR2 and TLR4 was also investigated, as these receptors have been shown to play an important role in recognition of parasite glycolipids, such as *P. falciparum* glycosylphosphatidylinositol (GPI) and *Leishmania* lipophosphoglycan (LPG), abundantly expressed on promastigote surface. When surface expression of TLR2 was analyzed (Figure 5b), a significant decrease was observed with *P. falciparum* (*P* < 0.05 with PfRBC 5 and 25), but the effect appeared to be partially nullified by the subsequent addition of LPS. When TLR2 mRNA levels were examined, no clear trend for any of the analyzed stimuli could be identified (Table S2), suggesting that the changes in cellular expression of TLR2 observed here may not have resulted from an enhanced or decreased gene transcription. Similarly, analysis of cellular and transcriptional expression of TLR4 after exposure to *L. donovani* and/or *P. falciparum* parasites did not highlight any clear effect from these stimuli on the receptor (data not shown).

### L. donovani promastigotes induce PS externalization at high doses

Pathogen-induced apoptosis of DCs has been described as a mechanism of immune dysfunction used by several micro-organisms, including *Plasmodium* spp., to evade adaptive immunity.\(^5\)\(^-\)\(^9\)

To investigate whether a similar event may account for the failure of our mo-DCs to mature and fully respond to LPS following exposure to *L. donovani* and *P. falciparum*, translocation of PS on the outer cell membrane was assessed by annexin V-FITC binding (Figure 6). Exposure of mo-DCs to the highest doses of *L. donovani* promastigotes resulted in a progressive rise in the proportion of PS-expressing cells (annexin V-positive), comparable to the effects induced by staurosporine, a commonly used initiator of apoptosis. Whilst this increase was evident (but not statistically significant) at the doses of 25 and 50 parasites/cell, no effect was displayed at lower parasite-to-cell ratios. On the contrary, *P. falciparum* and uninfected RBCs did not increase the number of annexin V-positive cells. When mo-DCs were co-exposed to the two parasites simultaneously, a more pronounced cytotoxicity appeared to affect the cells, as confirmed by the increased number of PS-expressing cells in samples stimulated with lower parasite-to-cell ratios. Similar results with *Leishmania* and/or *Plasmodium* parasites were obtained after a 24 and 48-h incubation (data not shown), providing evidence that the marked reduction in the number of gated cells observed at the highest parasite doses is consistent with an increased proportion of non-internalized parasites remaining in the sample (a fixed number of 10,000 events was measured per sample with non-internalized parasites being set out of the gate), rather than with a rise in the number of dead, down-sized (and as such not-gated) DCs, as it was in the case of staurosporine stimulation.
Figure 6. Dose-dependent apoptosis of mo-DCs cultured with *L. donovani* promastigotes, *P. falciparum*-infected RBCs or a combination of the two stimuli. Immature mo-DCs were cultured alone or in the presence of either *L. donovani* promastigotes, uninfected RBCs, *P. falciparum*-infected RBCs or *L. donovani* promastigotes and *P. falciparum*-infected RBCs at different parasite-to-cell ratios (ranging from 1:1 to 50:1) for 72 h. Thereafter, cells were harvested and expression of annexin V was measured by flow cytometry. Expression levels of annexin V are indicated in each scatter plot, whose data are representative of three independent experiments performed with mo-DCs from different donors. In the bar chart, the mean percentage of apoptotic cells ± SEMs (error bars) are shown for two independent experiments. *Ld* = *L. donovani*; *PfRBC* = *P. falciparum*-infected RBC; *Ld-PfRBC* = *L. donovani* and *P. falciparum*-infected RBC.
**Kinetics of mo-DC maturation inhibition and mo-DC apoptosis by *L. donovani* promastigotes**

To verify whether the lack of mo-DC maturation induced by high doses of *L. donovani* in the presence of LPS may be caused by a cytotoxic effect solely, expression of DC-SIGN, DC maturation markers (CD80 and CD86) and PS translocation were monitored at earlier time points (Figure 7). Pre-incubation of mo-DCs with increasing amounts of *L. donovani* promastigotes prior to LPS stimulation reduced the surface expression of DC-SIGN (*P* < 0.05 for *Ld* 25 and 50) and displayed an inhibitory effect on DC maturation, regardless of the duration of the pre-incubation step (Figure 7a). Thus, 1-h incubation with 25 or 50 *L. donovani* promastigotes per mo-DC inhibited the LPS-triggered expression of CD80 and CD86 to a comparable, dose-dependent extent as a 12-h or a 24-h incubation (*P* < 0.01 with *Ld* 25 and *P* < 0.001 with *Ld* 50 for CD80, *P* < 0.05 with *Ld* 50 for CD86) (Figure 7a).

When *L. donovani* promastigotes (parasite-to-cell ratio of 25:1) and LPS were added simultaneously to the mo-DCs, a reduced expression of DC-SIGN, CD80 and CD86 was already evident after 1-h incubation and became more visible after 12-h (Figure 7b) (*P* < 0.05 for CD80), confirming that the *Leishmania*-mediated interference of LPS maturation already starts at very early stages. When PS externalization was measured by annexin V binding, a dose-related increase above the baseline level (unstimulated cells) could be observed only after 24-h for *Ld* 25 and *Ld* 50, and after 48-h for all three doses tested (Figure 7c) (*P* < 0.05 for *Ld* 25 and *P* < 0.01 for *Ld* 50 after 48 h).

**Discussion**

In the present study, the effect of VL-malaria co-infections on DC function was explored by examining the activation of mo-DCs co-exposed to increasing concentrations of *L. donovani* promastigotes and *P. falciparum*-infected RBCs. The obtained data indicate that co-stimulation with low amounts of parasites results in the induction of partially anergic DCs, characterized by semi-mature phenotypes and progressive failure to release IL-12p70. Similar outcomes were observed in the presence of *L. donovani* only, in contrast to the more silencing *P. falciparum*, suggesting a dominating effect of *Leishmania* during co-stimulation.

In absence of serum opsonization, *Leishmania* parasites are generally believed to be devoid of DC-activating signals, as DCs incubated with either promastigote or amastigote stages have been shown to retain an immature phenotype and produce little IL-12p70, although with variable effects upon the different DC subsets and *Leishmania* species.\(^{16,17,19}\) Nevertheless, earlier studies conducted with bone marrow-derived DCs have demonstrated that whereas the majority of cells remained immature following infection with *L. major*, a small, but consistent fraction of DCs containing parasites or parasite debris slowly up-regulated the expression of maturation markers, like CD86, CD40 and HLA-DR.\(^{25,53}\) A similar scenario seems to apply to our model, as moderate up-regulation of CD80 and CD86 levels following *L. donovani* exposure was observed in a restricted proportion of mo-DCs only. This indicates that the *Leishmania* entry, or the presence of its intracellular forms, may not have remained completely silent in these cell populations. Importantly, we found that this partial activation of DCs was accompanied by a significant down-regulation in IL-12p70 release.
and by an enhanced IL-10 secretion, suggesting these DCs may act by preferentially priming IL-10-producing CD4+ T cells, deleterious for disease resolution. Our results, however, contrast with those of earlier studies showing release of IL-12 immediately following DC contact with *L. donovani*\(^{15,16}\) despite being in agreement with others in which *L. donovani* promastigotes failed to induce IL-12p70 secretion from DCs.\(^{18,19}\) Failure to detect IL-12p70 in our culture supernatants could also have resulted from its autocrine effects upon mo-DCs via constitutively expressed IL-12 receptors,\(^{54}\) which might explain the semi-mature phenotype detected in a fraction of these cells. Future work will have to prove the actual capacity of these DCs to stimulate proliferation of T cells and drive their differentiation towards regulatory T cells rather than Th1 cells. In contrast to this low-dose *Leishmania*-driven induction of semi-mature, IL-10-producing DCs, *P. falciparum* appeared to partially inhibit the slightly activating effects triggered by the uninfected RBCs, most likely caused by their allogeneic source, and only induced a marginal increase in co-stimulatory molecule expression. Lack of *in vitro* DC maturation upon *Plasmodium* exposure is a well-described phenomenon,\(^{33,34}\) although low parasite doses have been recently shown to induce slight up-regulation of DC maturation markers,\(^{34}\) in full compliance with our results. Interestingly, this *P. falciparum*-mediated inhibition appeared not to affect the phenotypic pattern triggered by the *Leishmania* parasites in co-stimulated DCs. Mutually exclusive internalization of the two parasites as a conceivable explanation was ruled out as remnants of *P. falciparum*-infected RBCs were found to co-localize with *L. donovani* parasites at cellular level. In addition, DC contact with *L. donovani*, but not *P. falciparum*, resulted in a sharp, dose-dependent reduction in the surface expression of DC-SIGN, not attributable to transcriptional down-regulation, confirming the strong role played by this C-type lectin in DC-*Leishmania* interactions.\(^{55,56}\) TLR2 expressed on monocytes/macrophages and natural killer cells has been shown to mediate recognition of *P. falciparum* GPI as well as *Leishmania* surface LPG.\(^{57,58}\) Therefore, we investigated whether internal competition for TLR2 occupancy or parasite-driven effects on its expression may have accounted for the *Leishmania*-driven priming of DCs during co-stimulation. In agreement with some previous findings,\(^{59-61}\) we found that *L. donovani* parasites were able to slightly increase the surface presence of TLR2 in a dose-dependent fashion, although transcriptional profiling did not yield clear results. Whilst these data are limited to draw conclusions on whether this parasite-mediated effect may or may not have influenced DC response to *L. donovani* and *P. falciparum* co-exposure, they provide evidence that malaria failure to inhibit *Leishmania*-driven priming of mo-DCs did not result from the leishmanial parasite avidity towards TLR2 nor from a paucity in its expression.

Inhibition of LPS-induced DC maturation by either *L. donovani* promastigotes, *P. falciparum*-infected RBCs or a combination of the two was shown to be dose-dependent. Whilst low parasite amounts (ranging from 1 to 5) did not affect LPS-induced maturation of mo-DCs, higher parasite-to-cell ratios resulted in a progressively increasing inhibition of LPS effects which intensified when both *L. donovani* and *P. falciparum* were present. Intriguingly, a similar pattern was observed for the proportion of cells displaying PS translocation, suggesting a close association between the two phenomena. Incubation with *L. donovani* at ratios of 25 and 50 promastigotes per DC triggered a pronounced increase in the expression of annexin V and enhanced, although not significantly, the percentage of PS-expressing cells, conceivably with the suppressing activity
Chapter 5

**DC-SIGN**

- LPS
- 1h pre-stimulation
- 3h pre-stimulation
- 5h pre-stimulation
- 12h pre-stimulation

**CD80**

**CD86**

**Annexin V**

- 5h
- 12h
- 24h
- 48h
DC maturation during VL-malaria co-infections

Figure 7. Kinetics of mo-DC maturation inhibition and mo-DC apoptosis by *L. donovani* promastigotes. (a) Immature mo-DCs were cultured alone or in the presence of *L. donovani* promastigotes at different parasite-to-cell ratios (ranging from 5:1 to 50:1) for different time intervals (ranging from 1-h to 12-h), followed by 48-h of incubation in the presence or absence of LPS (20 ng/mL). Thereafter, cells were harvested, and expression of DC-SIGN, CD80 or CD86 was measured by flow cytometry. (b) Immature mo-DCs were cultured alone or in the presence of *L. donovani* promastigotes (parasite-to-cell ratio of 25:1) and LPS (20 ng/mL) for different time intervals (ranging from 1-h to 12-h). Thereafter, cells were harvested, and expression of DC-SIGN, CD80 or CD86 was measured by flow cytometry. (c) Immature mo-DCs were cultured alone or in the presence of *L. donovani* promastigotes at different parasite-to-cell ratios (ranging from 5:1 to 50:1) for different time intervals (ranging from 5-h to 48-h). Thereafter, cells were harvested, and expression of annexin V was measured by flow cytometry. For charts a and b, data represent means + SEMs (error bars) of two independent experiments performed with mo-DCs from different donors. For chart c, data represent means + SEMs (error bars) of three independent experiments performed with mo-DCs from different donors. For all chart, data are indicated as the percent fold-change of expression vs. the unstimulated mo-DCs (100%), calculated by pooling the percent fold-change of each individual experiment. *Ld* = *L. donovani*. *P* <0.05 and **P* <0.01 for values that are significantly different from the unstimulated mo-DCs. *P* <0.05, ***P* <0.01 and ****P* <0.001 for values that are significantly different from the LPS-stimulated mo-DCs.

displayed on their maturation. On the contrary, *P. falciparum*-infected RBCs were able to induce a similar effect only at higher amounts (50 parasites per DC). Incubation with both parasites, on the other hand, exacerbated the cytotoxic effect elicited by the two stimuli, resulting in an increased, but statistically non-significant proportion of PS-expressing cells at parasite-to-cell-ratios of 5:1 and above. Apoptosis as a driver of unresponsiveness to LPS stimulation in mo-DCs exposed to high (50-100 parasites per DC), but not low (10 parasites per DC), doses of *P. falciparum* has been previously reported and our results fully comply with the respective literature. However, as PS translocation can also be apoptosis-independent, an ultimate role for this cell death mechanism as a result of DC-*P. falciparum* interactions remains to be confirmed. Conversely, an intrinsic ability to delay apoptosis induction in various cell subsets, including macrophages, mo-DCs and polymorphonuclear neutrophil granulocytes, has been described for promastigotes and amastigotes of several *Leishmania* spp., supporting the speculation that the *Leishmania* parasite may deliberately manipulate host cell apoptotic machinery to improve its survival and pathogenicity. This activity, however, arose when cells were challenged with a pathogen multiplicity equal to or lower than 10 parasites per cell, disregarding the potential cytotoxic effect that higher parasite numbers may display. Natural transmission models currently suggest the *Leishmania* inoculation size to vary between 10 and 100,000 promastigotes per sand fly bite. This makes it difficult to establish the precise physiological ratio of promastigotes in the spleen or liver, where DCs are likely to be co-exposed to *Leishmania* and *Plasmodium* parasites. The same challenge applies to *P. falciparum*-infected RBCs, although the recent finding that circulating DCs from individuals harboring low-level *P. falciparum* infection and CD8+ splenic DCs harvested from *P. chabaudi*-infected mice displayed apoptotic features clearly supports a role for the programmed cell death in malaria *in vivo* infections. While further studies are required to establish the physiological
relevance of the observations highlighted by the present study, particularly with respect to the use of amastigotes as a better proxy for the study of VL-malaria interactions and of in vivo-resembling parasite levels, the possibility that the DC apoptosis observed in the presence of large numbers of parasites may have resulted from culture medium exhaustion, as previously suggested, should not be excluded. Indeed in the present model, both parasites were used at concentrations (2.5-5 X 10^7 parasites/mL for ratios of 25-50 parasite per DC) that largely exceed the densities found in standard, long-term cultures. Interestingly, a dose-dependent inhibition of LPS-mediated DC phenotypic maturation by *L. donovani* promastigotes began to appear at very early stages, prior to the translocation of PS on the outer membrane layer, suggesting that specific mechanisms rather than a generalized toxicity may be responsible for this differentiation impairment.

In conclusion, we found that *L. donovani* promastigotes exerted a dose-dependent effect on mo-DCs differentiation, which ranged from semi-mature cells producing low IL-12p70, but high IL-10 levels to a complete lack of phenotypic maturation in the presence of high parasite doses. The effect preceded the onset of apoptosis and was displayed against unstimulated as well as LPS-stimulated cells. Conversely, *P. falciparum*-infected RBCs failed to activate mo-DCs and only slightly interfered with their LPS-triggered activation. When mo-DCs were stimulated with both parasites simultaneously, cells displayed an overall phenotype reflecting the *Leishmania* trigger. *L. donovani* was shown to co-localize with *P. falciparum* in the same DCs, interact with them via the C-type lectin DC-SIGN and slightly increase cellular expression of TLR2, important for DC recognition of surface glycolipids. This suggests that in the presence of a VL-malaria co-infection, *Leishmania*-driven effects may overrule the more silent response elicited by *P. falciparum*, shaping host immunity towards regulatory patterns and possibly delay resolution of the two diseases. Additional testing exploring the effect of asynchronous *L. donovani* and *P. falciparum* co-stimulation on various DC subsets, including ex vivo DCs isolated from tissues that are likely to co-encounter the two parasites or their products, such as blood, spleen or liver, will be required to support this conclusion and provide new insights into the immunobiology of VL-malaria co-infections.

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Author's contributions

EvdB, HMdB, PPSB, IvD and HDFHS conceived and designed the experiments. EvdB, HMdB and PPSB performed the experiments. EvdB, HMdB, PPSB, IvD and HDFHS analyzed the data. EvdB, HMdB, PPSB, PFM, ERA, MPG, IvD and HDFHS interpreted the data. EvdB, HMdB, PPSB, PFM, ERA, MPG, IvD and HDFHS wrote and approved the final version of the manuscript.

Competing interests

The authors have no competing interests.
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DC maturation during VL-malaria co-infections


Figure S1. *L. donovani* promastigotes promote semi-mature, anergic IL-10-producing moDCs that interfere with LPS-triggered activation.
Figure S2. *P. falciparum* fails to activate moDCS and partially interferes with LPS-triggered activation.
Figure S3. *L. donovani* drives activation of moDCs co-stimulated with *L. donovani* promastigotes and *P. falciparum*-infected RBCs, by promoting semi-mature, anergic IL-10-producing moDCs that interfere with LPS-triggered activation.

Figure S4. *L. donovani* alone or in combination with *P. falciparum* interacts with DC-SIGN.
Table S1. *L. donovani* alone or in combination with *P. falciparum* does not affect DC-SIGN transcriptional levels.

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<th>CD209 mRNA</th>
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<td><strong>Medium</strong></td>
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<td><em>Ld</em> 5</td>
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Table S2. *L. donovani* alone or in combination with *P. falciparum* does not affect TLR2 transcriptional levels.

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