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# IL-12p70 Production by *Leishmania major*-Harboring Human Dendritic Cells Is a CD40/CD40 Ligand-Dependent Process

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Leishmaniasis, a vector-borne parasitic disease, is transmitted during a sandfly blood meal as the parasite is delivered into the dermis. The parasite displays a unique immune evasion mechanism: prevention of IL-12 production within its host cell, the macrophage (i.e., where it differentiates and multiplies). Given the close proximity of skin dendritic cells (DC) to the site of parasite delivery, their critical role in initiating immune responses and the self-healing nature of *Leishmania major* (Lm) infection, we examined the interaction between myeloid-derived human DC and Lm metacyclic promastigotes (infectious-stage parasites) to model the early “natural” events of infection. We found that DC can take up Lm and, after this internalization, undergo changes in surface phenotype suggesting “maturation”. Despite the intracellular location of the parasite and resultant up-regulation of costimulatory and class II molecules, there was no detectable cytokine release by these Lm-harboring DC. However, using intracellular staining and flow cytometry to analyze cytokine production at the single-cell level, we found that Lm-harboring DC, but not monocytes, produce large amounts of IL-12p70 in a CD40 ligand (CD40L)-dependent manner. Finally, DC generated from mononuclear cells from patients with cutaneous leishmaniasis (Lm), once loaded with live metacyclic promastigotes, were found to reactivate autologous primed T lymphocytes and induce a CD40L-dependent IFN- $\gamma$  response. Our results link the required CD40/CD40L interactions for healing with DC-derived IL-12p70 production and provide a mechanism to explain the genesis of a protective T cell-mediated response in the face of local immune evasion within the macrophage at the site of *Leishmania* delivery. *The Journal of Immunology*, 2000, 164: 5858–5865.

**L**eishmaniasis is a vector-borne parasitic disease with clinical manifestations ranging from local cutaneous lesions to life-threatening visceral disease (kala azar), which are attributed to biologic differences among leishmanial species as well as among humans. However, in all *Leishmania* species, the infectious stage of the parasite, the flagellated metacyclic promastigote (1), enters the dermis after a sandfly blood meal. Once the metacyclic promastigote enters the host cell (macrophage), it differentiates into the intracellular stage, the amastigote, which divides and subsequently infects other cells, thus sustaining parasitism and subsequent transmission to other sandflies.

Skin-dwelling APC, specifically epidermal Langerhans cells (LC)<sup>3</sup> and dermal dendritic cells (DC), are actively involved in the surveillance of their environment (2, 3). These cells use phagocytosis, macropinocytosis, micropinocytosis, and receptor-mediated endocytosis (4) to sample their environment and capture many types of exogenous molecules and microorganisms. Data conflict on the ability of DC to take up *Leishmania* parasites, in particular

on whether or not the promastigote can enter DC. LC and DC within cutaneous lesions are reportedly parasitized by *Leishmania major* (Lm) in vivo in both human cutaneous leishmaniasis (5–7) and experimental murine cutaneous leishmaniasis (8, 9). In humans with cutaneous leishmaniasis, parasites (or their breakdown products) have been found in and around LC in biopsy specimens. Additionally, murine LC have been shown to take up *Leishmania* parasites and migrate to local draining lymph nodes for T cell activation (10). Another in vitro study reported that although primate and human epidermal LC do not take up stationary-phase leishmania promastigotes, dermal macrophages do under similar conditions (11). More recently, it has been shown that murine fetal skin-derived DC (FSDDC) are preferentially infected by Lm amastigotes compared with metacyclic promastigotes (12), whereas mouse CD11c<sup>+</sup> splenic DC take up Lm promastigotes (13).

In this study, immature cytokine-derived myeloid DC (driven by GM-CSF and IL-4) cultured from human peripheral blood were used to assess early events occurring at the site of delivery of *Leishmania* and to examine their ability to activate/drive T cells along a type 1 pathway and initiate immunity. We also evaluated the ability of the infectious stage of Lm (metacyclic promastigotes) to be taken up by human mononuclear cell-derived DC and detailed the events pursuant to that process. The data show that as a consequence of the interaction between Lm metacyclic promastigotes and DC, IL-12p70 can be produced in a CD40L-dependent manner. Furthermore, these parasite-harboring DC can process Lm metacyclic promastigote-derived molecules and present their processed products to human T cells from the blood of patients with local cutaneous leishmaniasis.

## Materials and Methods

### Patients and donors

DC were generated from buffy coats obtained from normal volunteer blood donors at the National Institutes of Health (NIH) Clinical Center,

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<sup>3</sup> Abbreviations used in this paper: LC, Langerhans cell; DC, dendritic cell; Lm, *Leishmania major*; FSDDC, fetal skin-derived DC; rhGM-CSF, recombinant human GM-CSF; CD40LT, CD40 ligand trimer; SAC, *Staphylococcus aureus* Cowan 1 bacteria; LPG, lipophosphoglycan.

Department of Transfusion Medicine. The four patients in this study with localized cutaneous leishmaniasis were seen at the NIH Clinical Center for diagnosis and treatment. They had no previous therapy and had the onset of ulceration ~2–3 mo before presentation. The time of presentation and ulcerated appearance of the lesions were typical for cutaneous leishmaniasis. All lesional biopsies were culture positive for *Leishmania*. Subsequent species confirmation of each isolate was performed, and all were typed as *Lm*. Informed consent was obtained from each of the patients.

### Parasites

*Lm* clone V1 (MHOM/IL/80/Friedlin) was cultured in 199 medium supplemented with 20% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, 40 mM HEPES, 0.1 mM adenine (in 50 mM HEPES), 5  $\mu$ g/ml hemin (in 50% triethanolamine), and 1  $\mu$ g/ml 6-biotin (M199/S). Infective-stage metacyclic promastigotes of *Lm* were isolated from stationary culture (4–5 days old) by negative selection using peanut agglutinin (Vector Laboratories, Burlingame, CA) as previously described (14). Amastigotes were isolated from footpad lesion homogenates of BALB/c mice by differential centrifugation and were cryopreserved. Parasites tested below the detection limits for endotoxin (<0.1 endotoxin units/ml, LAL assay; BioWhittaker, Walkersville, MD) and tested negative for mycoplasma (PCR detection method; American Type Culture Collection, Manassas, VA). Before infection of DC, parasites were opsonized with 5% normal human serum by incubation at 37°C for 30 min.

### Reagents, cytokines, and Abs

Recombinant human GM-CSF (rhGM-CSF) ( $1 \times 10^7$  U/mg) and rhIL-4 ( $2 \times 10^6$  U/mg) were obtained from PeproTech (Rocky Hill, NJ). rhIFN- $\gamma$  (500 U/ml) was obtained from PharMingen (San Diego, CA). mAbs to the following molecules were obtained commercially: CD14 (63D3) and glycophorin (10F7) (American Type Culture Collection); mouse IgG1 FITC and mouse IgG1 PE (Dako, Glostrup, Denmark); mouse IgG2b FITC, mouse IgG2b PE, CD56-PE (B159), CD3-FITC, and CD86-PE (B70/B7-2) (PharMingen); CD56-PE (Leu19), CD14-PE (Leu-M3), HLA-DR-PE, and CD80-PE (BB1/B7) (Becton Dickinson, San Jose, CA); CD1a-PE (Caltag, San Francisco, CA); CD1a-FITC (OKT6; Ortho Diagnostic Systems, Raritan, NJ); CD54-FITC (ICAM-1; Amac, Westbrook, ME); and CD19-FITC (Immunotech, Marseilles, France). mAbs to the following molecules were obtained as generous gifts: CD40 ligand trimer (CD40LT) and anti-CD40LT M90 (Immunex, Seattle, WA); CD19 (FMC63; H. Zola, Woman's & Children's Hospital, Adelaide, Australia); CD16 (3G8; J. Jaffe, Hahnemann University, Philadelphia, PA); CD3 (OKT3) (Orthoclone; Dr. Fucello, Ortho-Biotech, Raritan, NJ); and anti-lipophosphoglycan-FITC (LPG WIC-79.3; D. L. Sacks, National Institute of Allergy and Infectious Diseases). *Staphylococcus aureus* Cowan 1 bacteria (SAC) (1/10,000 dilution, Sansorbin) was obtained from Calbiochem (La Jolla, CA).

### Generation and characterization of immature DC

Cells were prepared exactly as previously reported (15), using the IL-4 and GM-CSF protocol for generation of large numbers of immature DC from peripheral blood-derived monocytes (16). All cells were cultured in RPMI 1640 (BioWhittaker) supplemented with 2 mM L-glutamine, 10 mM HEPES, 50 mM 2-ME, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% heat-inactivated FCS (Life Technologies, Gaithersburg, MD). All tissue culture media were found to be free of endotoxin (to the detection limits of the LAL assay). Briefly, PBMCs were isolated from normal blood bank donors or patients with cutaneous leishmaniasis using Ficoll-diatrizolate (Lymphocyte Separation Medium; ICN Biochemicals, Aurora, OH) centrifugation separation. The mononuclear cells were suspended at  $5 \times 10^6$  cells/ml and incubated for 2 h at 37°C under 5% CO<sub>2</sub>. The nonadherent cells were removed with gentle rinsing. The full volume of culture medium was replaced, and rhGM-CSF and rhIL-4 (1000 U/ml) were added. These cells were then cultured for 7 days, adding half-volume fresh media and full-volume fresh cytokines every other day. The DC were purified using two rounds of negative selection to remove lineage marker positive cells (anti-CD3 at 1:150; anti-CD14, anti-CD16, and anti-CD56 at 1:200; and anti-CD19 at 1:1000) with either magnetic cell sorting technique, magnetic cell separation system microbeads (Miltenyi Biotec, Sunnyvale, CA), or Dynal (Lake Success, NY) goat anti-mouse IgG beads as follows. The cells were suspended in ice-cold HBSS/5% FCS at  $10^7$  cells/ml with negative-selection Ab and were rocked at 4°C for 30 min, washed twice in HBSS/5% FCS, and suspended at  $2 \times 10^7$  cells/ml. The magnetic beads were added at 4:1 bead-to-cell ratio and incubated for 15 min (magnetic cell separation system) or for 30 min with rocking (Dynal) at 4°C. The cells were then placed onto a prepared column or into the magnetic field twice to enhance purity. The DC used in this study were routinely CD1a<sup>+</sup>

(>90%), HLA-DR<sup>+</sup> (>95%), CD86<sup>+</sup> (20%), CD40<sup>+</sup> (>95%), and negative for CD3, CD14, CD19, and CD56 by flow cytometry (FACSCalibur; Becton Dickinson).

Monocytes were isolated using a human monocyte isolation kit (Miltenyi Biotec) according to the manufacturer's guidelines after enriching for monocytes by cold aggregation of PBMCs (17) and were routinely >95% CD14<sup>+</sup> using flow cytometry.

### Coculture of DC/monocytes and *Lm* parasites

DC or monocytes were suspended at  $10^6$  cells/ml, and 0.3 ml of this cell suspension was placed into individual wells of 48-well plates (CoStar, Cambridge, MA). Opsonized parasites (ratio 4:1 per cell) were added to DC or monocyte cultures for 16–18 h at 37°C under 5% CO<sub>2</sub>. At the time of coculture, Polymixin B (10  $\mu$ g/ml; Sigma, St. Louis, MO) was added to the culture media for use in all wells. In experiments using CD40LT, the trimer was used at 1  $\mu$ g/ml. Cells and supernatants were pooled from triplicate wells to minimize well-to-well variability. Cell-free supernatants were collected after centrifugation and stored at –70°C until ELISA-based cytokine measurements were performed. Collected cells were washed four times with PBS and aliquoted for further analysis. Brefeldin A (10  $\mu$ g/ml; Sigma) was added during the final 6 h of culture when performing intracellular cytokine staining.

### Cytospin preparations

Cytospins were prepared using a Shandon II cytocentrifuge (Shandon Lipshaw, Pittsburgh, PA) set at 500 rpm for 5 min. After Wright-Giemsa staining, light microscopic analysis of the cells was performed in a blinded fashion, counting a minimum of 250 cells per slide to estimate both the infection rate and parasite number per cell. The cytopins were run in parallel with flow cytometry using anti-lipophosphoglycan (LPG)-FITC labeling.

### Flow cytometry

Cells were incubated with normal mouse sera to block nonspecific binding before staining. Staining was done in 100  $\mu$ l volumes with titrated amounts of directly conjugated mAb for optimal labeling. A minimum of 10,000 cells were acquired on a FACSCalibur and were analyzed using CellQuest Software (Becton Dickinson). For some experiments, the parasites were prelabeled with carboxyfluorescein diacetate (Molecular Probes, Eugene, OR) before coculture with DC, and then the DC were subsequently stained for surface molecules.

For intracellular cytokine staining, cells were fixed in 2% paraformaldehyde and washed with ice-cold PBS/0.1% BSA. The cells were blocked for 1 h or overnight in PBS/5% nonfat milk/0.1% saponin (Fischer Scientific, Pittsburgh, PA). The anti-IL-12-PE or mIgG1-PE isotype control Ab (PharMingen) was diluted (5  $\mu$ g/ml) in this same blocking buffer and added to the cells in a final volume of 50  $\mu$ l. Cells were stained for 30 min, washed twice with PBS/0.1% saponin, and then washed once with PBS alone. A minimum of 10,000 cells were analyzed as above. For specificity control, excess rhIL-12p70 (1  $\mu$ g) was coincubated with the Ab in the staining process.

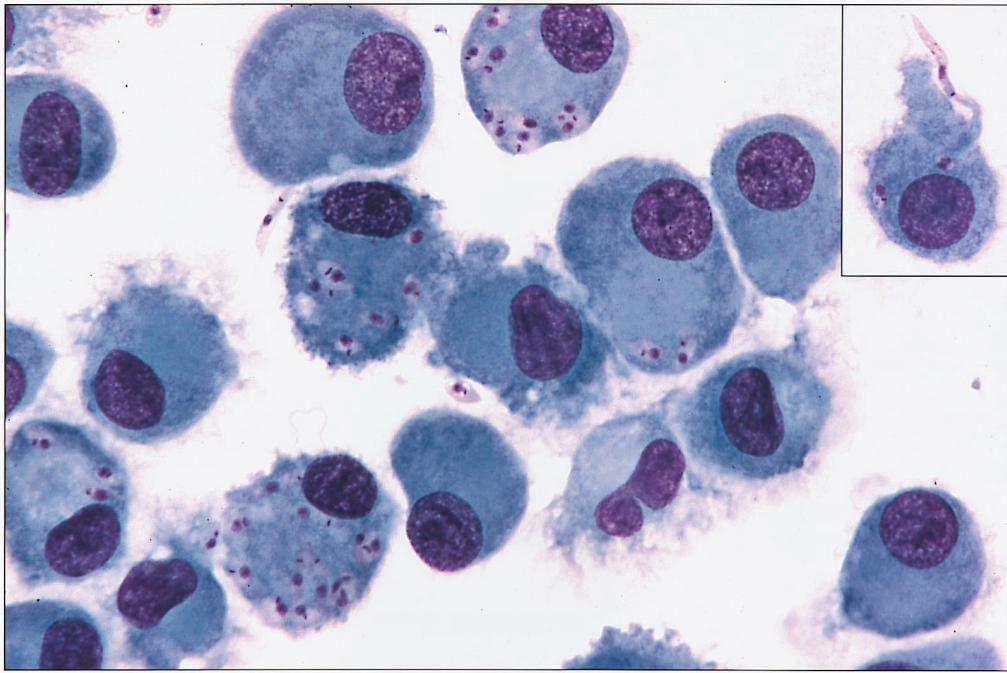
### ELISA-based cytokine detection assays

All cytokines were detected as secreted protein products in culture supernatants using cytokine-specific ELISA assays. Paired Abs were from R&D Systems (Minneapolis, MN; IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , and IL-6) and PharMingen (IL-12p70 and IL-10). Assays were performed according to the manufacturer's guidelines. The lower limits of detection for the assays were as follows: for IL-12p70, 31 pg/ml; for IFN- $\gamma$ , IL-1 $\beta$ , IL-6, and IL-10, 39 pg/ml; and for TNF- $\alpha$ , 48 pg/ml.

### Autologous T cell proliferation assays

DC were generated from *Lm*-infected patients. Some of these DC were infected overnight with *Lm* metacyclic promastigotes as described above and were washed extensively to remove any external parasites. Other DC were simply plated out in parallel but were not exposed to *Lm*. All DC were irradiated with 2000 rad before use in assays. Autologous T cells (responder cells) were isolated (Lympho-Quik T; One Lambda, Canoga Park, CA) and cocultured at  $10^5$  cells/well with varying concentrations of DC. The plates were incubated at 37°C under 5% CO<sub>2</sub> for 5 days, pulsed with 1  $\mu$ Ci/well of [<sup>3</sup>H]thymidine (DuPont, Boston, MA) for 16 h, and harvested onto filter mats for scintillation counting.





**FIGURE 1.** *Lm* metacyclic promastigotes are taken up by human DC. Human DC were incubated with *Lm* metacyclic promastigotes for 18 h. Cytospins were evaluated using light microscopy (magnification,  $\times 1000$ ) for infection rate and number of parasites/cell. Representative data from 1 of 21 experiments showing six infected cells in this field. Inset shows a metacyclic promastigote entering a DC.

#### Statistical analysis

Statistical analysis was performed using the Wilcoxon signed-rank test, and the correlation coefficient was determined using simple linear regression. All statistics were performed using StatView 5 (SAS Institute, Cary, NC).

## Results

### *Lm* metacyclic promastigotes enter monocyte-derived DC

To compare infection of DC with the two developmental stages that either initiate or maintain parasitism in the vertebrate host, DC were cultured with metacyclic promastigotes or amastigotes of *Lm* opsonized with human serum. After overnight incubation, supernatants were collected and cells harvested for further analysis. The infection rate with amastigotes was typically 1.5-fold greater than it was with the metacyclic promastigotes (average, 58 vs 38%), but the number of intracellular parasites was similar ( $\sim 3$  parasites/cell). Because we were interested in the initial events associated with the “natural route” of infection (i.e., the delivery of metacyclics into the dermis through the epidermis), the subsequent studies focused on DC infected with metacyclic promastigotes (Fig. 1). Most infected cells contain more than one parasite, and many of these intracellular forms are located peripherally within the cell. The inset in Fig. 1 shows a metacyclic promastigote entering a DC. Select DC + *Lm* metacyclic promastigote cocultures ( $n = 3$ ) were kept for 72 h and examined at 24 h intervals for parasite growth. Over the 3-day period, there was an approximate doubling of both the number of DC infected (from a mean of 38% at day 1 to 70% at day 3) and the number of intracellular parasites (from a mean of 3.3 organisms per cell at day 1 to 6.2 organisms per cell at day 3) within each infected cell.

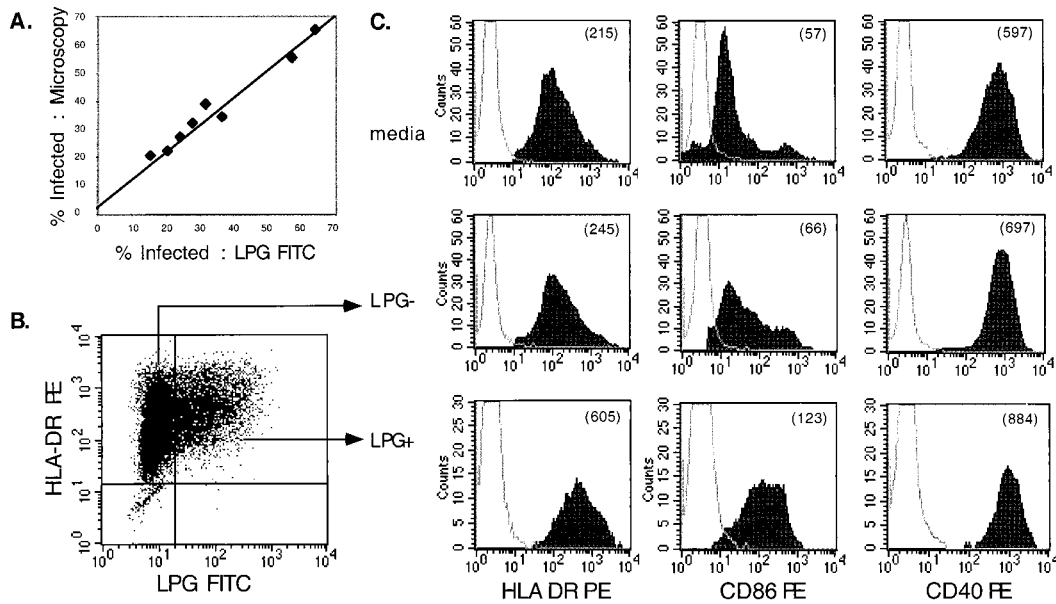
### *Lm*-harboring DC up-regulate HLA-DR, CD86, and CD40 surface molecules

To assess the effect of intracellular *Lm* on DC surface molecule expression, we labeled parasite-harboring DC (hereafter referred to as infected DC) using a mAb, anti-LPG FITC, that is specific for

the LPG-rich surface of metacyclic-stage parasites. We compared unexposed DC (media control) with parasite-exposed, uninfected DC (LPG<sup>-</sup>) and infected DC (LPG<sup>+</sup>). The distinction of infected and uninfected cells by flow cytometry was consistent with the microscopic analysis of stained cells (Fig. 2A). Fig. 2B shows a representative dot plot of an ungated DC population stained with both LPG-FITC and HLA-DR PE after overnight exposure to *Lm* metacyclic promastigotes. In Fig. 2C, unexposed DC (upper panels) and exposed populations (LPG<sup>-</sup>, uninfected, middle panels; or LPG<sup>+</sup>, infected, lower panels) with mean fluorescence intensities are shown. Compared with cells cultured in media alone, *Lm*-infected DC showed a 2.8-fold increase in HLA-DR surface expression, a 2.2-fold increase in CD86, and a 1.5-fold increase in CD40 in this representative experiment (minimum of four experiments). Similar results were obtained when the parasite was fluorescein-labeled before infection using carboxyfluorescein diacetate ( $n = 6$ ). In comparison to cells cultured in media alone, infected DC showed significant ( $p < 0.05$ ) average increases in surface expression of HLA-DR (3-fold), CD86 (2.4-fold), and CD40 (1.9-fold). Similar increases in these surface costimulatory molecules were observed when DC were infected with *Lm* amastigotes (data not shown).

### *IL-12p70* production by *Lm*-infected DC: a CD40/CD40L-dependent process

Although DC could be infected with *Lm* and, as a result, up-regulate surface accessory molecules, there were no cytokines detected in the overnight culture supernatants (IL-12p70, IL-1 $\beta$ , IL-6, IL-10, and TNF- $\alpha$ ; data not shown). Because CD40L is a known inducer of cytokines (including IL-12p70) in DC, we examined the effects of sequential additions of parasites and CD40LT on cytokine production (Fig. 3). To mimic the events that likely occur after entry of *Lm* into skin DC, including activation, maturation, and migration to the draining lymph node where they could receive the CD40L signal from primed T cells, CD40LT was



**FIGURE 2.** Lm metacyclic promastigote-infected DC express higher levels of HLA-DR, CD86, and CD40 surface molecules. *A*, Correlation between infected DC identified using LPG-FITC labeling (flow cytometry) and microscopic identification of infected DC ( $n = 8$ ;  $r = 0.97$ ). *B*, Dot plot showing an ungated, Lm-exposed population. *C*, *Upper panels* show unexposed DC (media control), *middle panels* show exposed but uninfected DC (gated on LPG<sup>-</sup>), and *lower panels* show infected DC (gated on LPG<sup>+</sup>) from a representative DC donor (of at least four). Shaded areas show staining for surface Abs, and isotype controls are unshaded.

added ~8–10 h after the parasites. As shown in Fig. 3A, no IL-12p70 was detected with media alone or with parasites alone (Lm). Although IL-12p70 production could be seen with CD40LT alone

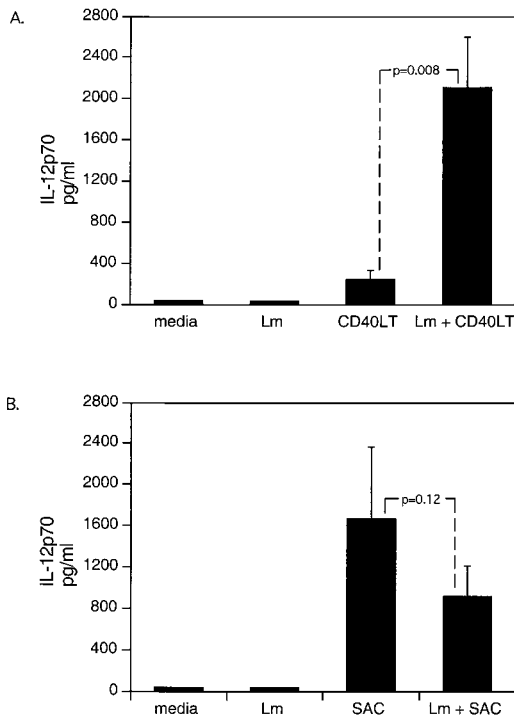
(242 pg/ml ± 88), there was a 10-fold increase in IL-12p70 when both Lm plus CD40LT were added (2108 pg/ml ± 489). In all donors tested ( $n = 13$ ), there was a significant increase in IL-12p70 production using both stimuli sequentially when compared with using either stimulus alone (CD40LT vs CD40LT + Lm,  $p = 0.008$ ; Lm vs CD40LT + Lm,  $p = 0.008$ ). Similar results were obtained with other cytokines including TNF- $\alpha$ , IL-6, IL-10, and IL-1 $\beta$  (data not shown). SAC, another potent inducer of IL-12p70, was tested in conjunction with Lm metacyclic promastigotes to determine whether there was any modulatory effect on IL-12p70 production by Lm. In seven donors tested, with sequential exposure to metacyclic parasites and then to SAC, there was no significant alteration in IL-12p70 production ( $p = 0.12$ ; Fig. 3B).

*Single-cell analysis shows that Lm-infected DC are a major source of IL-12 production*

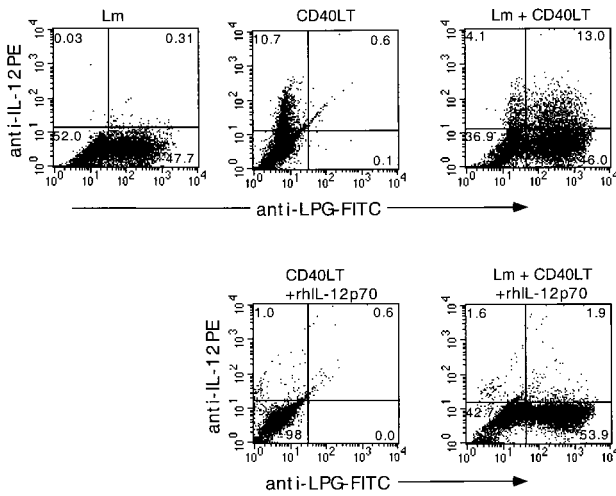
Using intracellular cytokine flow cytometry, IL-12 could be colocalized to LPG<sup>+</sup> DC (Fig. 4). In the absence of costimulation with CD40LT, infected DC did not produce IL-12. With CD40LT alone, 10.7% of cells stained for IL-12. When both Lm and CD40LT were added, ~22% of all Lm-infected DC stained positively for intracellular IL-12, and 76% of all IL-12<sup>+</sup> cells were Lm-infected cells. There was an augmentation of IL-12 production based not only on the cell frequency (1.5-fold increase) but also on the mean fluorescence intensity (1.6-fold increase) when comparing CD40LT alone to Lm plus CD40LT ( $n = 8$ ). This synergistic effect paralleled that seen in the secreted protein assays (not shown). The intracellular detection of IL-12 was specific with near complete blockade in the presence of exogenous rhIL-12p70.

*Lm-infected DC reactivate primed autologous T cells*

To determine whether DC can process live Lm metacyclic promastigotes and present the Lm-derived peptides (Ag) to sensitized T cells, DC were generated from monocytes recovered from Lm-infected patients; these DC were subsequently infected in vitro,

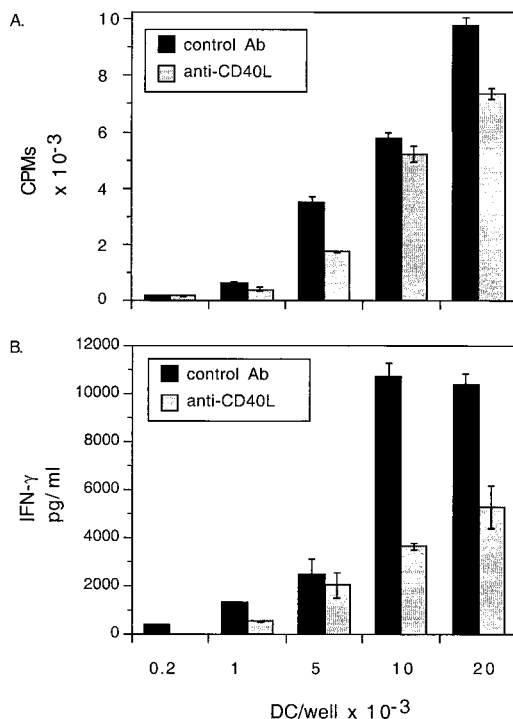


**FIGURE 3.** Lm-infected DC require a CD40/CD40L interaction to produce IL-12p70. DC were assessed for IL-12p70 production (mean ± SEM;  $n = 13$ ) in response to media alone, Lm metacyclic promastigotes, CD40LT, or Lm + CD40LT (*A*). *B*, The effect of Lm on SAC stimulation of DC is shown for comparison ( $n = 7$ ).

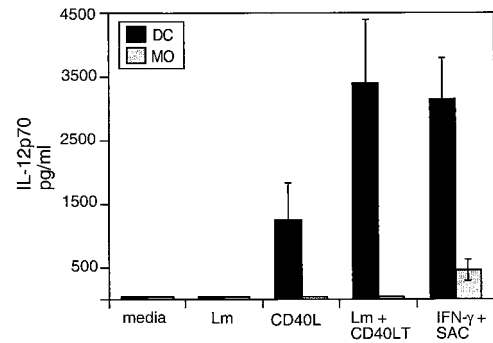


**FIGURE 4.** Single-cell analysis shows Lm-infected DC as a major source of IL-12 production. Flow cytometric analysis for intracellular IL-12 in Lm-exposed DC in response to Lm, CD40LT, and Lm + CD40LT (*upper panels*). rIL-12p70 was used as a specificity control and is shown in the *lower panels*. Numbers within the dot plots show the percentage of positive cells. The data are representative of eight experiments.

and autologous T cells were added ( $n = 4$ ) (Fig. 5). As the number of infected DC added to a fixed number of T cells was increased, there was an increase in [ $^3$ H]thymidine incorporation and IFN- $\gamma$  production compared with those T cells cultured with uninfected DC (Fig. 5). The IFN- $\gamma$  response was markedly inhibited by the



**FIGURE 5.** Lm metacyclic promastigote-infected DC reactivate autologous T cells from Lm-infected patients. Net [ $^3$ H]thymidine incorporation using DC prepared from patients with cutaneous leishmaniasis as APC and autologous T cells ( $10^5$ /well) in the presence (▨) or absence (■) of Ab to CD40L (A). B, IFN- $\gamma$  production by the same T cells. Data are expressed as mean  $\pm$  SEM and replicate cultures of a representative patient ( $n = 4$ ).



**FIGURE 6.** Differential effects of CD40LT on IL-12p70 production in DC compared with monocytes. IL-12p70 production (mean  $\pm$  SEM) in DC (■) and monocytes (▨) cultured in the presence of media alone, Lm alone, CD40LT alone, Lm + CD40LT, or IFN- $\gamma$  + SAC is shown using cells from four normal donors.

addition of an anti-CD40L Ab (Fig. 5B), an effect seen less dramatically when [ $^3$ H]thymidine incorporation was used as the read-out (Fig. 5A).

#### Differential effects of CD40LT on IL-12p70 production in Lm-infected DC vs monocytes

To test whether the effect of CD40LT was restricted to the induction of IL-12 in DC or if it could induce IL-12 in other APC exposed to Lm, we compared both DC and monocytes derived from the same normal donors ( $n = 4$ ) and exposed them to Lm metacyclic promastigotes (Fig. 6). With monocytes, there was no IL-12p70 detected in overnight supernatants using media alone, Lm metacyclic promastigotes alone, CD40LT alone, or the combination of Lm metacyclic promastigotes plus CD40LT. In marked contrast, DC from the same donors showed increased IL-12p70 in the presence of CD40LT alone and a significant 3-fold increase with the combination of CD40LT plus Lm under the same conditions. There were comparable infection rates in the monocytes and DC (64 vs 48%, respectively). The monocytes were capable of producing IL-12p70 when stimulated with IFN- $\gamma$  plus SAC, demonstrating that their capacity to produce IL-12p70 in response to a different stimulus was intact. Consistent with previous findings (18, 19, 40), when IFN- $\gamma$  plus SAC was used to stimulate Lm-infected monocytes (MO), no IL-12 was detected (in two of two donors tested: MO + IFN- $\gamma$  + SAC 936, 378 pg/ml; and Lm-MO + IFN- $\gamma$  + SAC < 39, <39 pg/ml of IL-12p70, respectively).

## Discussion

The present study demonstrates that human myeloid-derived DC can be infected with live, infectious-stage *L. major* promastigotes. As a result, there is an up-regulation of DC surface costimulatory molecules and CD40/CD40L-dependent IL-12p70 production. Use of this in vitro system allows dissection of the early immune response into its functional components, and it is used here to describe the events that might occur in the setting of natural *Leishmania* infection, once the parasite is delivered into a DC-rich environment (into the dermis through the epidermis). Because the tone of the immune response may be set early in the course of infection (and greatly influenced by the cytokine milieu), we focused our efforts on events occurring within the first 18 h after Lm exposure.

In this study we used human DC for three reasons: 1) their proximity to the site of delivery of the parasite by the sandflies; 2)



their potential ability to generate a protective IL-12-driven cell-mediated immune response, in part as a result of their ability to migrate to local draining lymph nodes; and 3) murine data suggesting alternative (nonmacrophage) cellular sources of IL-12. That DC may indeed be that IL-12 source is suggested by the well-described inhibition of IL-12 production within parasite-harboring macrophages in murine models (18–20), the observed *in vivo* production of IL-12 in response to *Leishmania* infection (21), and the absolute requirement for IL-12 to mediate healing even in a low-inocula model (22).

Using light microscopy and flow cytometry, we determined that metacyclic promastigotes were taken up by DC, the stage of the parasite that initiates parasitism in the vertebrate host. Our results are in agreement with murine studies using both Lm amastigotes and metacyclic promastigotes to infect FSDDC (12), splenic DC (13), or epidermal LC (9), although the present study suggests (for human DC at least) that infection with metacyclic promastigotes occurs more efficiently than has heretofore been appreciated. In contrast to these results, others have reported the inability to infect human or primate LC with stationary-phase promastigotes (11). Possible explanations for this discrepancy could be differences in the subpopulations of DC used, culture conditions, human serum opsonization, and the variability in the number of infectious-stage parasites (metacyclic promastigotes) when using stationary-phase vs purified metacyclic parasites.

The presence of Lm within DC resulted in an increase in HLA-DR, CD40, and CD86 surface expression, findings that suggest the DC were undergoing maturation (4). The up-regulation of these molecules was a direct consequence of infection, as indicated by two-color flow analysis of DC surface molecules and parasite Ag (Fig. 2). Using murine FSDDC infected with amastigotes, a similar increase in MHC expression, CD40, and CD86 has been described, although when metacyclic promastigotes were used, no surface changes were appreciated in bulk FSDDC populations (12). A similar absence of surface changes with exposure to the metacyclic stage has been recently reported using mouse spleen-derived DC (13). These conflicting data point to the importance of specifically identifying those cells infected with the parasite (Figs. 1 and 2) because when the frequencies of infected cells are small (12), significant changes are difficult to identify. Our phenotypic findings are supported by data using recombinant *Leishmania brasiliensis* Ag (LeIF)-treated human myeloid DC in which the expression of B7-1, CD40, and CD54 is increased (23).

Interestingly, we found that although the DC were clearly harboring Lm and had surface changes associated with activation/maturation, there were no cytokines produced (in particular IL-12) in the absence of appropriate costimulation. Because it has been reported that ligation of CD40 on the surface of DC is a key step in the induction of IL-12 production (24, 25), the role of the CD40/CD40L interaction in IL-12 production by Lm-infected DC was examined. Our study demonstrates a second signal requirement for IL-12p70 production by Lm-infected human DC and shows that CD40L meets this requirement. Further, using two-color analysis of intracellular cytokines and parasite LPG, the production of IL-12 by Lm metacyclic promastigote-infected DC in the presence of CD40L could be easily documented (Fig. 4). Therefore, we suggest that the DC is rendered capable of producing IL-12, once it is infected, only after receiving an additional signal provided by CD40L. DC infected with amastigotes (rather than metacyclic promastigotes) had similar requirements for IL-12 production. Indeed, in a number of parallel studies ( $n = 4$ ) comparing promastigotes to amastigotes, IL-12p70 production after amastigote infection was also CD40L-dependent. As with the promastigotes, DC surface CD86, CD40, and HLA-DR was increased after amastigote infec-

tion (data not shown). The idea that both stages have similar costimulatory requirements for IL-12 production may enhance the biological relevance of this finding because as the infection develops, the amastigote stage (the stage that maintains infection) is clearly more abundant and likely more accessible to peripheral DC.

The critical role for CD40/CD40L interactions has been demonstrated in the protective immune response in experimental leishmaniasis by using mice with the targeted disruption of CD40/CD40L (26–28). This protective response is related to IL-12 production (27, 29–32). Recently, several murine studies detected DC-derived IL-12p40 in response to *Leishmania* (*Leishmania donovani* (33) and Lm (12, 13)), a response that was not clearly CD40L-dependent; however, IL-12p70 production was detectable in only one of these studies. IL-12p70 secretion by human DC in response to a recombinant *Leishmania* Ag or *Toxoplasma* infection has been detected and, in each case, was in part CD40L-dependent (23, 34). The requirement for CD40L for IL-12p70 production in Lm-infected human DC may be beneficial from the parasite standpoint in that *Leishmania* is simply less likely to induce IL-12 unless there is sufficient and appropriate costimulation, a process that would most likely occur after DC migration to the draining lymph node. Other important costimulatory molecules have been studied in the setting of early leishmaniasis (35), although they were not directly studied for this article. However, the consistent increase in expression of CD86 (B7-2) and HLA-DR (MHC class II) molecules in DC harboring Lm parasites likely reflects their involvement in the immune response. In fact, the incomplete inhibition of the proliferative response (compared with cytokine production; see Fig. 5) by CD40/CD40L blockade speaks to additional, and more dominant, costimulatory requirements for proliferative responses.

The particular type of DC used in this study is a less mature DC, more equipped for capturing extracellular molecules/microorganisms for further processing than for immunostimulation (4, 36). This exact cell type has been shown to migrate to the T cell area of draining LN when generated *ex vivo* and reinfused *s.c.* in a primate model (37). Very clearly these DC, once infected, can reactivate primed T cells. Further, the Lm-infected DC can reveal the presence of IFN- $\gamma$ -secreting T cells, a process that is inhibited by blocking CD40L with an antagonizing monoclonal Ab. This inhibition of IFN- $\gamma$  production suggests a role for CD40L-driven IL-12p70 in recall immune responses to Lm metacyclic promastigotes.

Our data also suggest that Lm metacyclic promastigotes may exert different effects once inside DC vs monocytes. We show that although CD40L costimulation of Lm-infected DC induces large amounts IL-12, no IL-12 was induced in monocytes from the same donors under identical stimulation (Fig. 6). Indeed, specific inhibition of mononuclear phagocyte cytokine production has been well described in the setting of infection with *Leishmania* (in particular with metacyclic promastigotes) (18–20, 38–40). Alternatively, the observed differential effects of CD40L on DC vs monocytes may reflect the need for higher levels of costimulation or for additional stimuli to induce IL-12 in monocytes (41). Unlike what has been seen in murine macrophages (18–20) and in bulk human PBMC culture systems (40), Lm infection of human DC does not significantly inhibit SAC-driven IL-12 production (Fig. 3B).

Our data fit into the existing body of literature and may help to explain an apparent discrepancy regarding *in vitro* and *in vivo* findings. Although *in vitro* studies show that macrophage IL-12 production is clearly prevented by *Leishmania* metacyclic infection at both the mRNA and protein levels (18, 19, 39), infection of mice with Lm metacyclic promastigotes *in vivo* drives IL-12 production and a protective IFN- $\gamma$  response (21) and is an absolute

requirement for healing. Our in vitro results suggest that Lm-infected DC are capable of producing IL-12p70, given an appropriate second signal delivered through CD40.

The events described in this paper suggest that although the draining lymph node may be a site for initiation of IL-12-driven Th1 immune responses through Lm-infected DC, there may be concomitant IL-12 inhibition within the macrophage compartment operating at the site of the lesion (i.e., the site of parasite delivery and development). Additionally, overproduction of TNF- $\alpha$  and TGF- $\beta$  at the site of the lesion may contribute to the local pathology, inhibit healing (42, 43), and provide a temporary refuge for parasite growth.

Lending support to the importance of CD40-mediated induction of IL-12 is a recent study in a murine vaccine model in which a strong IL-12-dependent adjuvant effect of CD40LT DNA was seen when coadministered with soluble *Leishmania* Ag (44). This requirement for CD40L costimulation may give the parasite a temporal advantage, allowing peripheral amplification while the infected DC migrate to the draining lymph node, where they presumably engage in immunologic cross-talk with T cells (and perhaps NK cells). This DC-T cell interaction results in up-regulation of CD40L on clustered T cells and CD40 triggering on the DC (24).

The idea that the type of APC involved influences the nature of the ensuing immune response is one that has been previously addressed by others (45, 46) and is supportive of our data. In a BALB/c murine model, GM-CSF-treated bone-marrow-derived macrophages pulsed with Lm Ag have been shown to protect against a lethal challenge of *L. major*. This protection was contingent upon three factors: 1) specialized "activation" of the macrophages with GM-CSF (as M-CSF-treated and -untreated macrophages failed to protect), 2) IL-12 production, and 3) T cell contact. These GM-CSF-activated macrophages may resemble DC, as murine myeloid DC can be generated from GM-CSF-stimulated bone marrow-derived macrophages (47). Similarly, it seems likely that the requirement for contact with sensitized T cells involves CD40/CD40L interactions. Additionally, others have directly compared murine epidermal LC and macrophages that were pulsed with leishmania Ag and found that only the LC (not macrophages) could protect against challenge (48).

This in vitro model of parasite/human DC interactions can be readily adapted to any leishmania species. We have used it to delineate appropriate costimulatory requirements for IL-12p70 production, a prerequisite for inducing naive T lymphocytes to produce IFN- $\gamma$ . Ongoing efforts in our laboratory suggest that other leishmanial species (e.g., *Leishmania tropica*) differ from Lm once taken up by human DC (unpublished data). In the case of Lm, usually a self-healing cutaneous infection (49, 50), production of IL-12p70 by DC costimulated in the lymph node may be the force that drives a protective and healing immune response.

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