

The Differential *In Vitro* Presentation of *Mycobacterium leprae* Antigens by Human Dendritic Cells is determined by the Mechanism of Host Cell Adhesion

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Abstract

Clinical manifestations in leprosy, a chronic infectious disease caused by *Mycobacterium leprae*, depend on the immunologic reactions of the patient to the bacilli. Previously, we have shown a reversion of the inefficiency of the cellular-mediated immune response against *M. leprae* in lepromatous leprosy patients when dendritic cells (DCs) were used *in vitro* as APC. The aim of the current study is to investigate the cell-to-cell interaction when purified human monocytes-derived DCs and macrophages from healthy adult donors were co-cultured with autologous lymphocytes in the presence of either *M. leprae* or *M. bovis* BCG by using APCs which were either attached or in suspension. Subsequently, APCs were analyzed by optical microscopy, phenotypically monitored by flow cytometry, and cytokines secretion evaluated by ELISA and Luminex. Overall, our results suggest that: (1) the process of adhesion of the APC may not be absolutely essential for antigen presentation activity; (2) IL-12 production appears to be in direct relation to the susceptibility of the host cell to infection with *M. leprae*; (3) in the presence of autologous lymphocytes, there is a down-modulation of surface markers studied, except for CD209 in *M. leprae*-infected CD11c⁺ DCs, although a similar decreased expression of these markers on macrophages was also observed, and this step occurs in an antigen-dependent manner; and (4) the cytokines secretion is also dependent on the APCs' adhesion process. In parallel, the human THP-1 cell line was assessed showing that no monokine was differentially regulated when cells remained either attached or in suspension, regardless of the stimulus employed. These results may further guide future pivotal experimental procedures when studying *in vitro* cellular immune responses against mycobacteria.

Keywords: Leprosy; BCG; Antigen presentation; Monocyte; Cytokine, Cell adhesion

Introduction

Leprosy is a chronic infectious disease that primarily affects the skin, mucous membranes and nerves. Currently, 2 to 3 million individuals are infected with *Mycobacterium leprae* and the detection of new cases continues to increase [1]. The disease presents a spectrum of clinical manifestations that are directly related to a distinct immune response profile to the pathogen, and affects more than half a million cases per year [1-3].

The first crucial step in adaptive immunity is the activation of naive T cells by specific antigen presenting cells (APC). The importance of dendritic cells (DC) as sentinels of the immune system is emphasized, as they capture antigens at the site of infection and then migrate towards the lymph nodes [4-6,7].

Whether a Th-1 or Th-2 profile immune response occurs depends primarily on the nature of the APC. We previously demonstrated that monocyte-derived DCs, obtained from peripheral blood mononuclear cells (PBMC) of lepromatous leprosy patients, when stimulated *in vitro* with *M. leprae*, induced a Th-1 immune response, reversing the absence of a specific response to *M. leprae* primarily observed in these patients [8]. Furthermore, we also observed the acquisition of an *in vitro* immune response in patients who had Erythema Nodosum Leprosum (inflammatory manifestations in leprosy), through the expression of CD80 (highly expressed on DCs), a biomarker that represents the acquisition of a cell-mediated immune response in lepromatous leprosy [9].

DCs usually survey the immune system, and studies of interactions of DCs with T cells identified a novel receptor on DCs, named DC-SIGN or CD209 [10]. This receptor triggers the initial contact between

DCs and T cells via binding to the adhesion molecule ICAM-3 on T cells [10]. The identification of this receptor on DCs has been very useful in understanding the mechanism by which various pathogens cause infection [11-13].

DCs are a small subset of human PBMC that are potent stimulators of several T cell functions. In this context, several techniques for investigating DC functions have been developed [8,14,15].

Classically, most studies involving monocyte-derived DCs isolated from human blood initially purify the monocytes from PBMC through density gradients with subsequent culture of the whole PBMC by exploiting the ability of monocytes to attach to plastic substrates [16-22]. Thus, monocytes may be separated from the lymphocytes after 2 hours by culturing at 37°C, and the non-adherent lymphocytes can be removed. The adherent cells (monocytes) are then usually left untreated or treated with rIL-4 and rGM-CSF to differentiate these cells into macrophages or DCs, respectively [15]. However, this purification

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technique based on the adhesion properties of monocytes may not only reduce cell numbers and viability but also interfere with the expression of surface molecules, since the cells are harvested by scraping or by specific enzymes, such as trypsin, to allow flow cytometry assays. Other relevant considerations are: (1) The time-span needed to perform these procedures; and (2) The possibility of cell culture contamination, during the frequent handling processes.

Therefore, an easier alternative approach to monocyte purification from human PBMC, as well as subsequent culturing them in suspension, has previously been developed by our group for functional testing of monocytes-derived DCs and macrophages [8]. Following purification of PBMC by density gradients, cells are submitted to a "cold aggregation" step for half-hour, according to Mentzer et al. [22]. Subsequent examination of the cultures of aggregated cells in polystyrene tubes showed that they consisted of purified monocytes [8,9]. These monocytes remain in suspension and can be left untreated or treated with rIL-4 and rGM-CSF so that after 10 days of culture, monocytes-derived macrophages and DCs are successfully generated to follow up functional assays for monitoring the antigen presentation ability of these cells [8,9]. To our knowledge, the present paper is the first to compare the antigen presentation ability of monocytes-derived DCs and macrophages produced by the adhesion or aggregation-suspension techniques, using whole *M. leprae* and *M. bovis* BCG as stimuli. In parallel, the human monocytic cell line THP-1, which provides continuous culture and grows in suspension, was also used alone (none co-culture) in order to monitor the monokine secretion during *M. bovis* BCG infection. As part of this study, the major focus was on the expression of CD14, CD209 and CD11c, as well as the cytokines secreted in the supernatants from the cells.

Materials and Methods

Source of human mononuclear cells

The cells used here were obtained from leukocyte concentrate (buffy-coat), collected from healthy donor volunteers enrolled at the Hematology Unit from the Clementino Fraga Filho University Hospital (Federal University of Rio de Janeiro-UFRJ). This project is approved by the Ethics Committee of the Fluminense Federal University (CAAE-0126.0.258.000-09). For the establishment of the human acute monocytic leukemia cell line THP-1 (American Type Culture Collection, Rockville, MD, USA) in a macrophage-like state, cells were grown and expanded as previously described [23]. In brief, THP-1 cells were left for a whole 2 weeks in chamber slides (see below) with 1 ml cell suspension (1×10^6 cells) in each well. Differentiated, plastic-adherent cells were washed twice and rested in the culture medium, or infected with *M. bovis* BCG (see below) for 48 hours. For monocyte-like state, THP-1 cells were cultured in parallel in tubes, and then infected with *M. bovis* BCG for 48 hours.

Antigens

Irradiated *Mycobacterium leprae* was kindly donated by Drs. Patrick Brennan and John Spencer (Colorado State University, CO, USA); samples of lyophilized *Mycobacterium bovis* Bacillus Calmette Guerin (BCG Moreau strain) were kindly donated by Dr. Luiz R. Castello-Branco (Ataúpho de Paiva Foundation, Brazil) and used according to Simas et al. [24]. In some experiments, the mitogens phytohaemagglutinin (PHA, Oxford Immunotec, UK) or Concanavalin A (ConA, Sigma-Aldrich, St. Louis, MO, USA) were used at 1% aqueous final concentrations.

Isolation of human peripheral blood mononuclear cells (PBMC) and purification of monocytes

PBMC were isolated from the buffy coat by Ficoll-Hypaque gradient sedimentation [8,9,21]. Subsequently, monocytes were separated from lymphocytes by "cold aggregation", as developed by Santos et al. [8], based on the report of Mentzer et al. [18]. Briefly, 5×10^7 PBMC resuspended in DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with 10% FCS (Hyclone, Logan, UT, USA) were incubated during 30 min at 4°C under continuous rocking agitation. The monocytes were then precipitated spontaneously for 1 min on ice and the pellet containing the aggregated monocytes was washed in RPMI medium plus 10% FCS, and submitted to a new cycle of clumping under agitation at 4°C. After centrifugation at 238 g for 1 min at 4°C, the pellet was resuspended in fresh medium for culturing. The final pellet usually contains >80% of monocytes, as routinely monitored by flow cytometry and morphological analysis. A small contamination of CD3⁺ T cells was also detected (<5%). The supernatant obtained after the "cold aggregation" technique contains resting lymphocytes, and these cells were cryopreserved in liquid nitrogen until later use during interaction assays in co-cultures.

Differentiation of dendritic cells (DC) and macrophages

Purified monocytes were cultured in 12 × 75 mm polystyrene tubes which not allow the adhesion of the cells (Beckton Dickinson, Franklin Lakes, NJ, USA) standing up (vertical position) and LabTek® chamber slides (Nalge Nunc International, Rochester, NY, USA) at a concentration of 1×10^5 cells/ml in DMEM medium. The cells were untreated or incubated with rhGM-CSF (100 U/ml; Schering-Plough, Belgium) and rhIL-4 (1000 U/ml, Peprotech, Rocky Hill, NJ, USA) as described by Sallusto et al. [15] and modified by Santos et al. [8]. The differentiation of monocytes into DCs and macrophages was monitored by using anti-hCD209-PerCP staining (Beckton Dickinson, Franklin Lakes, NJ, USA) in a flow cytometry approach, according to several studies [8,9,22,25-27].

Interaction of monocytes-derived DCs or macrophages with *M. leprae* or *M. bovis* BCG with or without autologous lymphocytes

M. leprae 20 µg/ml or *M. bovis* BCG at 1 ng/ml was incubated with monocytes-derived macrophages or DCs at 37°C in 5% CO₂ incubator for 4 hours. Afterwards, autologous lymphocytes (1:7; APCs: lymphocytes ratio) at a concentration of 2×10^5 cells (chamber slides), and 1×10^6 cell (tubes) in DMEM medium were or were not added for additional 12 hours. Negative or baseline (medium only) and positive controls (PHA and ConA) were also set up in parallel. The cells cultured in chamber slides and infected with *M. leprae* or *M. bovis* BCG were fixed with 1% paraformaldehyde (PFA) and stained by Kynioun's acid-fast method (Dalynn Biologicals, Calgary, Canada), as described previously [28]. At the same time, 1×10^5 cells cultured in 100 µl of RPMI medium plus 10% FCS in polystyrene tubes and infected with *M. leprae* were centrifuged (424 g/5 min). The pellet was collected in specific tubes to be used in Cytospin 3 preparations (Shandon, Runcorn, Cheshire, UK). Afterwards, slides containing cytospin centrifuged cells were fixed and stained as the same procedure above. The cells were counted by using Optical Microscopy (400X), in 100 aleatory fields, and the results were expressed as percentage calculated as follow: [(number of cells infected by *M. leprae*-number of control cells/number of control cells) × 100]. *M. leprae* infection was observed by analyzing dark pink mycobacteria inside macrophages or DCs through the optical microscope Axioplan

2 microscope (Axio Observer 21, Zeiss, Germany). Cell-free culture supernatants were collected and stored at -70°C for further assays.

Flow cytometry

Protocols were used as before [8,9,25-27]. Briefly, the cells were washed with PBS plus 0.1% bovine serum albumin plus 0.01% sodium azide (PBS-FACS), and stained with $1\ \mu\text{g}/\text{ml}$ of anti-hCD14-PE (Becton Dickinson, Franklin Lakes, NJ, USA), and $10\ \mu\text{g}/\text{ml}$ of anti-hCD11c-FITC (ebioscience, San Diego, CA, USA) and anti-hCD209-PerCP on ice in the dark. After 30 min, cells were washed with PBS-FACS, resuspended in $500\ \mu\text{l}$ of 1% PFA and analyzed using a flow cytometry device (FACScalibur, Beckton Dickinson, Franklin Lakes, NJ, USA). Cell gate regions were drawn around viable cells based on their forward scatter (FSC) versus side scatter (SSC) properties in order to exclude cell debris. In double-staining experiments, an extra-independent circular cell gate region was also drawn around viable cells. In addition to detect the light scattered from the cells, FL-1(FITC) and FL-3 (PerCP) signals were set up. The results were expressed as the percentage of stained-positive bright cells.

ELISA

Detection of human interleukin (IL)-12 in supernatants was measured using a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Luminex

Detection of IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17, GM-CSF, G-CSF, monocyte chemoattractant protein 1 (MCP-1), macrophage inhibitory protein 1 (MIP-1 β), IFN- γ and TNF- α concentrations in duplicates by multiplex cytokine approach was performed by using the Bio-Plex protein multi array system, which uses Luminex-based technology as specified by the manufacturer (Bio-Rad, Hercules, CA, USA). Briefly, cell culture supernatants were thawed once and diluted 13-fold (except for baseline: 2-fold) in medium before being assayed. All analyte determinations were performed with the same batch of reagents.

Statistical analysis

For data in the tables, numbers of samples analyzed are presented in parentheses. The results are expressed as percentages with means, along with standard deviations. Statistical analysis used the student t test and ANOVA for comparison. Data were deemed to be significantly different at P values ≤ 0.05 .

Results

Microscopical evaluation

Protocols for monocytes purification from human PBMC through cold aggregation and afterwards, culturing them in polystyrene tubes to follow different experimental designs were first developed by Santos et al. [8]. Although this protocol has been routinely used in our facility, the present paper is the first to validate a comparative study between the two techniques: (1) attached cells (cells cultured in chamber slides) vs. (2) cells in suspension (cells cultured in polystyrene tubes).

Using the Santos et al. [8] protocol monocyte-derived macrophages and DCs infected with *M. leprae* and *M. bovis* BCG were photographed with bacteria staining pinkish after the fuchsin reaction (Figure 1A-1H). The morphological characteristics of DCs cultured in chamber slides were striking, with dendritic membrane projections forming a network (Figure 1E). This network was not

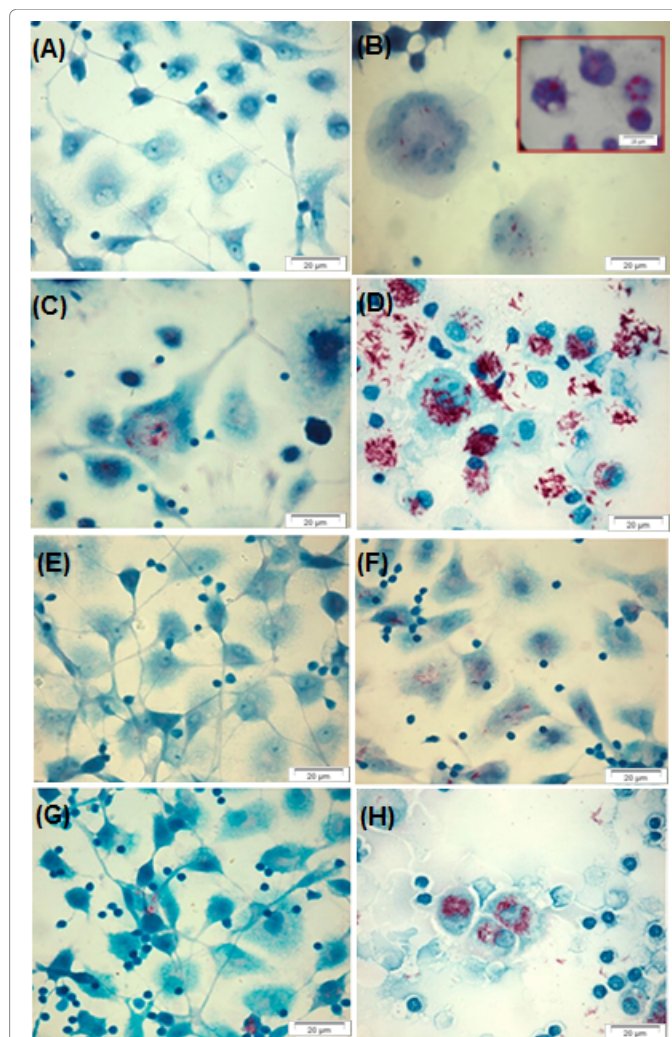


Figure 1: Optical microscopy analysis of *M. leprae* and *M. bovis* BCG interaction with attached antigen presenting cells. Optical microscopy employing Kinyoun staining after 16 hours of interaction of *M. leprae* and *M. bovis* BCG with (A-D) human monocytes-derived macrophages and (E-H) human monocytes-derived dendritic cells (DC) isolated from peripheral blood. (A and E) baseline, (B and F) cells infected with *M. leprae*, (C and G) cells infected with BCG, and (D and H) cells infected with *M. leprae* and BCG. Bar: $20\ \mu\text{m}$; Photomicrographs of monocyte-derived macrophages heavily infected with *M. leprae* (B) and *M. bovis* BCG (C) depicted in pinkish color. Interestingly, macrophages infected with *M. leprae* formed syncytia (multinucleate cells; see "B"). Also, monocyte-derived dendritic cells (DC) infected with *M. leprae* and *M. bovis* BCG are depicted in pinkish color. It is noteworthy that the striking morphological characteristic of DCs, with evidence of membrane projections like "dendrites", is the formation of a network.

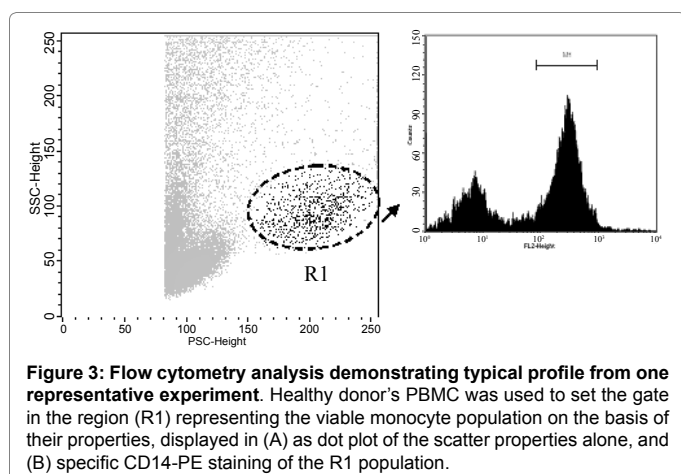
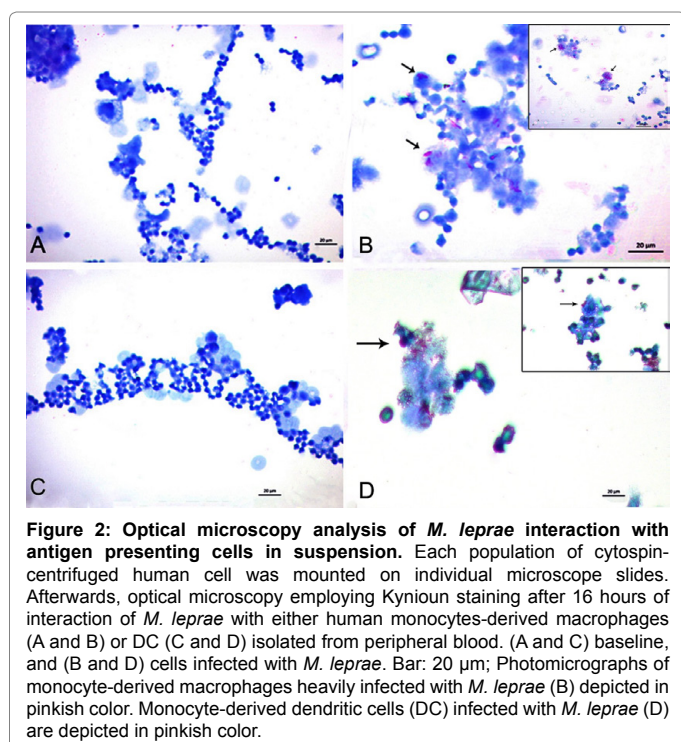
developed when DCs were simultaneously infected with *M. leprae* and *M. bovis* BCG (Figure 1H). Interestingly, macrophages infected with *M. leprae* formed syncytia (multinucleate cells) after 16 hours of interaction (Figure 1B).

Monocyte-derived DCs were infected by *M. leprae* and *M. bovis* BCG (Figures 1F and 1G, respectively); however, rates of infection of macrophages were more enhanced by these pathogens (compare Figures 1B and 1C, respectively). However, morphological characteristics of DC and macrophages cultured in polystyrene tubes (Figure 2) were quite different when we compare to the morphological aspects observed in cells that were cultured in chamber slides (Figure 1). After cytospin process, both macrophages and DC in suspension

displayed a rounded aspect, once the morphology may have distorted, resembling the peripheral blood monocytes. It's also interesting to notice that, both macrophages and DC in suspension, were permissive to *M. leprae* infection at comparable rates (rate of infection: 64% and 60%, respectively).

Flow cytometry evaluation

Figure 3 represents a typical cellular profile analyzed by flow cytometry, in which the characteristics of light scattering (FSC vs. SSC) were used to distinguish each cell population, and also to define the setting used further to assess APCs and lymphocytes specific staining. The different profiles were obtained for each condition of culture analyzed: cells in the tubes were slightly different from cells in the chamber slides (data not shown). In the latter, a population typical of monocytes (Mo) was evident and therefore the cells were also analyzed in the R1 gate.



Flow cytometry analysis of floating APC cultures in the absence of autologous lymphocytes.

Initially, the phenotypic study on mycobacteria-infected APC cultures was only performed in tubes, and not in chamber slides (Table 1). As expected [5], at baseline conditions monocyte-derived DCs showed nearly double the expression of CD209 when compared to macrophages ($38.8\% \pm 1.0$ vs. $18.7\% \pm 11.2$, respectively). On the other hand, when stimulated with *M. leprae* alone, macrophages had twice the CD209 level compared to the baseline condition, as did DCs in CD11c/CD209 markers when stimulated with *M. bovis* BCG (Table 1). As a whole, this set of experiments found very consistent data, similar to conventional, attached APCs cultures.

Differential expression of CD209 in *M. leprae*-infected CD11c DCs co-cultured with autologous lymphocytes

When autologous lymphocytes were added to the tube system, there were simultaneous decreases in all APC surface markers (around 30-fold at the highest magnitude in *M. bovis* BCG-infected DCs for CD209), with the exception of CD209 in mycobacteria-infected CD11c+ DC (Table 2). Tables 2 and 3 compare the phenotypic analysis on APCs of mitogen-stimulated and mycobacteria-infected cultures in the presence of autologous lymphocytes when cells were kept floating and attached, respectively. Strikingly, the expression of CD209 displayed higher levels ($p < 0.05$) in *M. leprae*-infected attached DCs when compared to both macrophages and baseline conditions (Table 3), as well as to *M. leprae*-infected CD11c+ DC alone in suspension (Table 1). Importantly, the specific induction appeared to be lymphocyte driven, because negative modulation of CD209 was found in both macrophages and DCs (Tables 1 and 2). Notably, CD11c and CD209 showed very similar profiles in both culture systems, regardless of the condition and APC analyzed.

Also noticed was the parallel, although predictable, increase of CD14 expression on macrophages (around 6-fold at the highest magnitude) in all conditions tested when cells were cultured in chamber slides and tubes (Tables 1 and 3).

In sum, surface markers remained very homogeneous in expression between both culture systems and respective conditions, except for *M. leprae*-infected CD11c/CD209 DCs in tubes as previously stated.

Cytokines detected by ELISA and Luminex

During intra-assay comparison, no significant difference was found for the *in vitro* IL-12 production detected by ELISA (Figure 4A) or Luminex (data not shown) when autologous lymphocytes were co-cultured with APCs either in tubes or in chamber slides. Remarkably, internal evaluation showed the highest IL-12 levels ($p < 0.05$) in those *M. leprae*-infected DCs which were cultured in chamber slides, when compared to macrophage counterparts (Figure 4B).

However, higher levels of IL-8 (9-fold increase) and MIP-1 β were found in *M. bovis* BCG-infected PBMC in the attached system only (Table 4). For MIP-1 β specifically, there was an almost 21-fold increase at the highest magnitude. For G-CSF, this stimulus induced higher levels in both culture systems: almost 2.6-fold and 3.6-fold increased secretion for chamber slides and tubes, respectively. On the other hand, a decrease in production was shown by IL-10 (from 2.5- to 8-fold), IL-17 (almost a 3-fold) and MCP-1 when BCG infected the PBMC in both culture systems (Table 4). For MCP-1 only, this stimulus reduced the secretion in both culture systems: almost 750-fold and 2,500-fold lower levels for chamber slides and tubes, respectively. For IL-4 and IFN γ , a 2-fold lower level was detected in the chamber slides only.

Surface marker	Baseline		<i>M. leprae</i>		<i>M. bovis</i> BCG	
	MΦ	DC	MΦ	DC	MΦ	DC
CD11c	64.1 ± 2.6 ^a	56.0 ± 1.8	60.8 ± 0.2	63.5 ± 14.4	71.7 ± 13.3	44.0 ± 17.2
CD14	57.0 ± 5.9	37.6 ± 8.1	45.9 ± 11.3	20.0 ± 5.7	33.3 ± 0.8	27.5 ± 13.0
CD209	18.7 ± 11.2	38.8 ± 1.0	34.6 ± 0.1	30.6 ± 22.6	23.3 ± 13.2	36.6 ± 21.2
CD11c/CD209	1.2 ± 0.2	2.0 ± 0.6	1.6 ± 0.9	0.2 ± 0.2	0.0 ± 0.0	4.0 ± 2.9

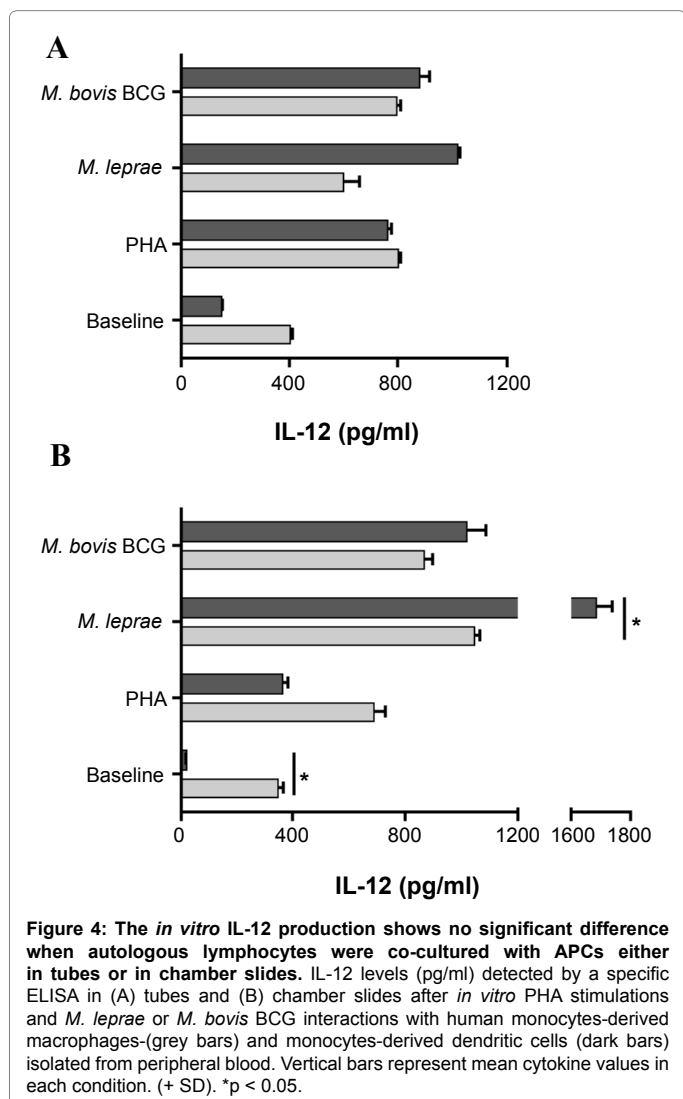
^aMean ± SD

Table 1: Phenotypic markers (%) of macrophages (MΦ) and dendritic cells (DC) from normal individuals after purified, differentiated and infected with either *M. leprae* or *M. bovis* BCG for additional 16 hours.

Surface marker	Baseline		PHA		<i>M. leprae</i>		<i>M. bovis</i> BCG	
	MΦ	DC	MΦ	DC	MΦ	DC	MΦ	DC
CD11c	19.5 ± 9.1 ^a	23.6 ± 11.9	15.1 ± 14.0	22.1 ± 11.0	18.2 ± 8.0	22.7 ± 8.3	17.0 ± 14.9	23.6 ± 7.3
CD14	14.6 ± 10.4	7.4 ± 5.9	32.2 ± 18.0	8.0 ± 2.4	23.8 ± 20.0	13.8 ± 2.6	20.2 ± 20.0	10.3 ± 9.3
CD209	5.6 ± 2.8	2.9 ± 0.5	7.4 ± 5.0	3.2 ± 1.9	2.9 ± 0.9	2.5 ± 0.2	1.9 ± 1.5	1.3 ± 1.3
CD11c/CD209	3.4 ± 2.2	1.6 ± 1.3	9.6 ± 9.0	5.4 ± 3.0	10.9 ± 5.7	1.7 ± 0.8	11.0 ± 5.6	5.0 ± 4.5

^aMean ± SD

Table 2: Phenotypic markers (%) of macrophages (MΦ) and dendritic cells (DC) from normal individuals after purified, differentiated, stimulated with the mitogen phytohaemagglutinin (PHA) and infected with either *M. leprae* or *M. bovis* BCG, and co-cultured with autologous lymphocytes for additional 16 hours in polystyrene tubes.



Positive controls were used to force cytokines secretion in PBMC cultured in the chamber slides only, and those mitogens have shown

consistent differences. Thus, PHA was stronger stimulus than ConA for IL-1β, IL-6, IL-8 and G-CSF productions. Curiously, ConA induced almost 5-fold higher MIP-1β levels when compared to PHA. Conversely, no difference was found for the *in vitro* IL-4, IL-10, IL-17, GM-CSF, IFNγ and MCP-1 productions when positive controls were compared to corresponding baseline levels.

Finally, when the THP-1 cell line was assessed, only IL-1β and IL-8 showed striking differences when the chamber slides were employed (Table 5). The adhesion process itself induced spontaneous secretion, because *M. bovis* BCG did not change the baseline scenario, which consisted in THP-1 cell line exposed with the culture medium alone. Virtually no production was detected for any monokine studied when cells remained in suspension in the tubes, regardless of the stimulus. Similar data were obtained in shorter (24 hours) interaction (data not shown). Together with IL-12p70, we were not able to detect IL-5, IL-7 and IL-13 productions by Luminex in the supernatant of either tubes or chamber slides (data not shown).

Discussion

The methodology of culturing human mononuclear cells floating in polystyrene tubes has been used regularly (first reported in [8]); however, to the best of our knowledge, virtually no report concerning the assessment against typical chamber slide in the context of neglected tropical diseases has been published to date. Thus, we have performed a comparative analysis employing a flow cytometry approach in a characteristic phenotypic profiling study on lymphocytes and monocyte-derived APCs stimulated with either *M. leprae* or *M. bovis* BCG. Two different techniques were used to maintain cells as either attached or floating. In some experiments, we have also used ELISA and Luminex in order to detect cytokine secretion. The optical microscopy confirmed both mycobacteria infections, as well as the visual branched projections, which are featured in the DC population.

Remarkably, our optical microscopy results proved that both *M. leprae* and *M. bovis* BCG-infected human APCs are quite vulnerable to the pathogens, although macrophages were more susceptible than DCs. In fact, attached DC secreted higher IL-12 levels than macrophage, and therefore, DC may be activated to produce reactive nitrogen and oxygen intermediates (RNI and ROI, respectively) to control the infection [5]. Both DC and macrophages, when cultured in polystyrene tubes (cells

Surface marker	Baseline		PHA		<i>M. leprae</i>		<i>M. bovis</i> BCG	
	MΦ	DC	MΦ	DC	MΦ	DC	MΦ	DC
CD11c	20.5 ± 3.8 ^a	19.3 ± 8.0	20.7 ± 9.0	19.4 ± 4.2	22.9 ± 4.3	17.0 ± 12.8	21.7 ± 4.3	20.7 ± 6.4
CD14	17.8 ± 15.5	2.6 ± 1.7	18.4 ± 15.8	8.6 ± 6.0	18.1 ± 8.1	10.8 ± 3.8	12.7 ± 10.0	4.0 ± 2.9
CD209	2.6 ± 1.6	2.1 ± 0.8	3.0 ± 0.5	3.0 ± 0.4	3.4 ± 1.7	36.1 ± 9.0 ^{b,c}	1.7 ± 0.3	1.6 ± 1.3
CD11c/CD209	3.5 ± 2.8	1.6 ± 1.2	8.1 ± 6.1	7.6 ± 2.5	3.4 ± 2.4	30.0 ± 6.0 ^{b,c}	3.8 ± 3.7	2.5 ± 1.1

^aMean ± SD; ^{b,c}p < 0.05, when compared to corresponding baseline and between cultures, respectively.

Table 3: Phenotypic markers (%) of macrophages (MΦ) and dendritic cells (DC) from normal individuals after purified, differentiated, stimulated with the mitogen phytohaemagglutinin (PHA) and infected with *M. leprae* or *M. bovis* BCG, and co-cultured with autologous lymphocytes for additional 16 hours in chamber slides.

Condition		Cytokines												
		IL-2	IL-4	IL-6	IL-8 ^a	IL-10	GM-CSF	IFNγ	TNFα	IL-1β	IL-17	G-CSF	MCP-1	MIP-1β
PBMC Slide	Baseline	51.7	22.7	397.8	75.3	9.2	114.6	416.1	70.2	24.5	2.2	24.5	5,375.2	471.0
	PHA	106.5	38.4	21,342.7	444.6	9.2	220.9	687.7	123.6	139.3	2.2	135.3	3,754.9	742.0
	ConA	169.2	35.4	1,545.3	169.1	9.2	184.2	531.9	128.8	61.2	2.2	35.2	4,078.5	3,426.6
PBMC Slide	Baseline	116.7	32.1	6,813.3	139.1	23.2	180.9	515.6	90.2	242.5	8.0	125.3	5,230.3	625
	<i>M. bovis</i> BCG	115.2	16.7	4,638.4	1,215.1	9.2	118.1	270.3	144.6	289.2	2.2	326.5	2.1	13,112.9
PBMC Tube	Baseline	105.1	38.0	10,433.8	156.4	72.9	202.2	528.1	125.2	492.3	5.1	227.6	3,291.5	25,746.4
	<i>M. bovis</i> BCG	136.7	28.4	8,388.8	231	9.2	153.4	406.6	201.9	491.7	2.2	795.1	4.4	28,419.0

^ang/ml, otherwise pg/ml.

Table 4: Cytokine levels in supernatants of PBMC cultures stimulated with the mitogens phytohaemagglutinin (PHA) or Concanavalin A (ConA) for 24 hours, or infected with *M. bovis* BCG for 48 hours, and detected by a multiplex array system.

Condition		Monokines								
		IL-6	IL-8 ^a	IL-10	GM-CSF	TNFα	IL-1β	G-CSF	MCP-1	MIP-1β
THP-1 Slide	Baseline	34.3	8.5	9.2	5.5	4.5	145.2	24.5	2.1	2.8
	<i>M. bovis</i> BCG	34.3	10.8	9.2	5.5	4.5	177.4	24.5	2.1	2.8
THP-1 Tube	Baseline	34.3	0.005	9.2	5.5	4.5	3.1	24.5	2.1	2.8
	<i>M. bovis</i> BCG	34.3	0.005	9.2	5.5	4.5	3.1	24.5	2.1	2.8

^ang/ml, otherwise pg/ml.

Table 5: Monokine levels in supernatants of *M. bovis* BCG-infected THP-1 human cell-line cultures for 48 hours and detected by a multiplex array system.

in suspension), have shown a rounded aspect (probably due to cytospin process), which is characteristic of fresh peripheral blood monocytes.

Extraordinarily, in this experimental condition, although DC releases higher IL-12 levels than macrophages, there are superior IL-12 levels induced in *M. leprae*-infected DC in attached cells only. It is noteworthy that although the latter condition is higher in the *M. leprae*-infected macrophages in both experimental adhesion conditions, the IL-12 levels are much higher when DC is attached. Consequently, the IL-12 production appears to be directly related to the susceptibility of the host cell to infection with *M. leprae*. And this fact, perhaps, corroborates the similar degree of infection with *M. leprae* comparable in both macrophages and DC in suspension, unlike the increased susceptibility to infection with *M. leprae* seen in adherent macrophages, when compared to adherent DC.

It is worth noting that the *M. leprae* batch used here was irradiated. Recently, the literature reported a comparative *in vivo* and *in vitro* effect of irradiated vs. live *M. leprae*, demonstrating that *M. leprae* induced lipid bodies' formation in host cells. The outcome was independent of the viability of the mycobacteria [29]. We have produced convincing data showing that fresh monocytes were successfully cultured in both chambers (attached) and tubes (floating). A good example to illustrate this model is the blood vessel, where cells are activated by numerous stimuli in the circulation, and migrate to a remote location of a given tissue upon receiving a specific inflammatory signal.

Following comparison of the culture system, we assessed both APCs co-cultured with autologous lymphocytes in the presence or

absence of mycobacteria. As a whole, equivalent expressions were regularly present, regardless of stimuli. In one scenario only, a subset of DC expressing CD11c showed a significant increase of CD209 that may be result of a positive modulation induced by *M. leprae*-activated lymphocytes in the attached system. On the other hand, a similar high CD209 level was found in those cultures lacking lymphocytes, but not in the CD11c+ DC population. Merely by exclusion, the culture system is expected to interfere with that expression, since higher CD11c/CD209 levels were found in the chamber slides only. This suggests that the adhesion process is important for CD209 expression, at least for *M. leprae*-infected monocyte-derived DCs. Alternatively, in those assays performed in tubes with stimulated-macrophages only, the CD14 expression was dependent on the APC source, similar to that previously reported for attached APCs [27-30]. However, when lymphocytes were added, an aberrant CD14 expression was typically evident.

A characteristic profile on cells that were kept floating suggests that apoptosis may be implicated in reducing the number of viable cells (unpublished observations), as well as the phenotypic profile displayed in the *M. leprae*-infected cultures. Thus, it is tempting to speculate that the lymphocytes in tubes might be presenting a cytotoxic profile against *M. leprae*-infected DC. Although this is not novel, we believe that the majority of the cell population from adult, otherwise healthy donors in our endemic setting is already sensitized to the antigens of the pathogens employed in this study.

The CD209 expression on DC represents the high affinity receptor for ICAM2 and ICAM3 by binding to mannose-like carbohydrates [31].

When we analyzed the APCs in the absence of autologous lymphocytes, the CD209 expression was normally expressed. As reported earlier, the CD209 induction on macrophage is a crucial step after *M. leprae* infection. This receptor, concomitantly with the absence of ROI and RNI, supports intense proliferation of the mycobacteria inside the APC, as seen in lepromatous leprosy lesions [5,32].

The detection of IL-12 during the interaction of APC and lymphocytes in the presence of both *M. leprae* and *M. bovis* BCG confer antigen-presenting ability to both macrophage and DC, regardless of whether the APC is adherent or floating. It is noteworthy that the highest IL-12 levels found in *M. leprae*-infected monocyte-derived DCs in chamber slides mirrored the increased expression of CD209, which was also detected in that same condition and system. IL-12 is preferentially produced by activated DC and its production is associated with the activation of NF- κ B [33,34]. Additionally, IL-12 promotes cellular immunity and protection against intracellular pathogens by means of commitment of a Th-1 phenotype [35-37]. After appropriate stimulation, roughly 25% of the blood monocytes are estimated to differentiate to DC in the circulation, but the majority may mature in tissue macrophages [38,39]. After infection with *M. bovis* BCG, *M. tuberculosis* or *M. leprae*, mononuclear phagocytes rapidly produce cytokines, such as IL-1 β , TNF- α , IL-6, IL-10 and IL-12 [40-44]. Curiously, monocytes-derived DCs pretreated with *M. bovis* BCG were significantly impaired in IL-12 p70 production [44].

Concerning the other cytokines studied, we showed here that production of IL-10, IL-17 and MCP-1 decreased in both *M. bovis* BCG-infected adherent and floating PBMC. Conversely, previous reports found that *in vitro* IL-10 was significantly up-regulated in adherent human macrophages during leptospiral infection [45]. Those results were in line with prior reports that leptospiral glycolipoprotein induces IL-10 production of human PBMC. However, data cannot be directly compared or extrapolated due to different pathogens employed among studies. On the other hand, our study detected lower levels of IL-4 and IFN γ in adherent cells only. Interestingly, the immunization of BCG-vaccinated individuals with MVA85A increased antigen-specific IFN- γ -secreting T cells concomitantly with the induction of antigen-specific IL-17A secreting cells and a decrease in CD4+ CD25+ CD39+ Treg cells [46]. In the leprosy context, the presence of Th-17-related cytokines reduced the levels of FoxP3+ Treg cells with simultaneous enhancement of IL-17 producing CD4+ cells [47].

More recently, Zhang et al. [48] reported that *M. bovis* BCG induced the production of MCP-1, RANTES, IL-12, TNF- α and IL-6 by DCs, while (virulent) *M. bovis* induced secretion of IL-1 β , IL-10 and IL-23. Furthermore, *M. bovis*-infected DCs induced CD4+ T cells to express higher levels of IL-17, a Th-17-type cytokine, while *M. bovis* BCG-infected DCs stimulated higher production of IFN γ , a Th-1 type cytokine. In another study performed by Kirkaldy et al. [49], the *in vivo* expression of MCP-1 and RANTES were again significantly elevated, but there was no difference in the mRNA expression of IL-8 in leprosy skin lesions. In addition, there was no significant difference in the expression of CCR2, CCR5 and CXCR2.

Strikingly, in our study the adhesion process was critical for the secretion of IL-8 and MIP-1 β . Both factors were promptly induced by attached macrophages infected with *M. bovis* BCG, but not in cell suspension. However, the same stimulus induced high levels of G-CSF, regardless of the system employed. In line with our data, Xue et al. [45] found in a recent study that both IL-8 and MIP-1 β were up-regulated *in vitro* in adherent human macrophage, in which the different mRNA or protein regulation was a pathogenic *Leptospira*-specific chemokine.

This could indicate that the phagocyte would attract other leukocytes to the sites of leptospiral infection more efficiently.

Concerning the THP-1 cell line, the adhesion process itself induced spontaneous IL-1 β and IL-8 secretion; the *M. bovis* BCG infection did not change the scenario. On the other hand, we were not able to detect any monokine when cells remained floating, regardless of the stimulus. Surprisingly, Novikov et al. [50] found that *M. tuberculosis* but not *M. bovis* BCG, consistently stimulated higher IL-1 β secretion in PMA-adherent THP-1 cell cultures in a similar fashion to primary macrophages. In keeping with our cell line findings, that secretion in our study was also determined by a multiplex assay.

Taken together, we conclude that: (1) There are evident morphological differences between monocyte-derived macrophages and DCs (adherent cells) displaying dendritic projections on the DC plasma membrane. This probably reflects a greater flexibility of this population to present antigen to T cells, mainly through the IL-12 production, when compared to the macrophages; (2) Monocyte-derived macrophages and DC (cells in suspension) have shown a similar morphology as the one seen in fresh peripheral blood monocytes; (3) An important outcome of this work is the demonstration, for the first time, that the antigen presentation ability of APCs to lymphocytes had no significant difference when analyzed by two experimental conditions, i.e. tubes or plates (cells in suspension or adherent cells, respectively); and (4) Finally, the method of culturing monocytes in order to differentiate them into either macrophages or DC in suspension, for long periods of time and for subsequent analysis in flow cytometer, is very promising. This result points out to the necessity of additional functional studies to investigate APCs and lymphocytes interactions in several pathological conditions.

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