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Mycobacterium leprae induces a tolerogenic profile in monocyte derived dendritic cells via Toll-like receptor 2 induction of Indoleamine 2,3-dioxygenase

Running title: TLR2 induces IDO-1 expression in human dendritic cells

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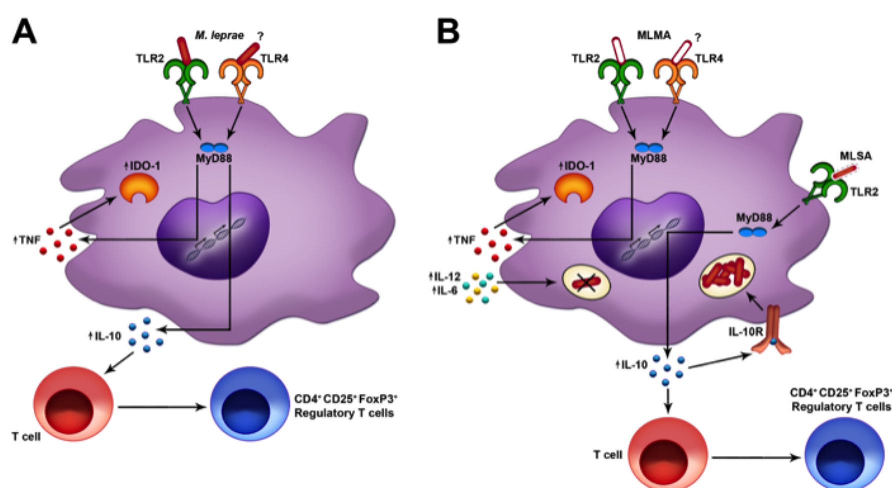
Abstract

The enzyme Indoleamine 2,3-dioxygenase (IDO-1) is involved in the first stage of tryptophan catabolism and has been described in both microbicidal and tolerogenic microenvironments. Previous data from our group have shown that IDO-1 is differentially regulated in the distinctive clinical forms of leprosy. The present study aims to investigate the mechanisms associated with IDO-1 expression and activity in human monocyte-derived dendritic cells (mDCs) after stimulation with irradiated *Mycobacterium leprae* and its fractions. *M. leprae* and its fractions induced the expression and activity of IDO-1 in human mDCs. Among the stimuli studied, MLMA induced the production of proinflammatory cytokines TNF and IL-6 whereas MLSA induced an increase in IL-10. We investigated if TLR2 activation was necessary for IDO-1 induction in mDCs. We observed that in cultures treated with a neutralizing anti-TLR2 antibody, there was a decrease in IDO-1 activity and expression induced by *M. leprae* and MLMA. The same effect was observed when we used a MyD88 inhibitor. Our data demonstrates that co-culture of mDCs with autologous lymphocytes induced an increase in regulatory T (Treg) cell frequency in MLSA-stimulated cultures, showing that *M. leprae* constituents may play opposite roles that may possibly be related to the dubious effect of IDO-1 in the different clinical forms of disease. Our data show that *M. leprae* and its fractions are able to differentially modulate the activity and functionality of IDO-1 in mDCs by a pathway that involves TLR2, suggesting that this enzyme may play an important role in leprosy immunopathogenesis.



M. leprae and its membrane fraction (MLMA) are able to induce IDO-1 via TLR-2 activation. The induction of IDO-1 by *M. leprae* in mDCs is associated with increased IL-10 levels and contributes for the expansion of $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells (A). In contrast, the activation of IDO-1 by MLMA is accompanied by an increase in pro-inflammatory cytokines like TNF, IL-6 and IL-12 and may be associated with a microbicidal effect (B). The soluble fraction of *M. leprae* increases IL-10 and $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells by an IDO-1-independent mechanism (B)>

Introduction

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Leprosy is a chronic infectious disease, which mainly affects the peripheral nerves and skin. The disease is caused by *Mycobacterium leprae*, an obligate intracellular parasite that predominantly infects macrophages, endothelial cells and Schwann cells, being the unique specie of mycobacteria capable of infecting peripheral nerves (1).

Previous studies have demonstrated that *M. leprae* induces an increase in the gene and protein expression of the enzyme Indoleamine 2,3-dioxygenase (IDO-1) in human monocytes (2). IDO-1 is an intracellular enzyme that catalyzes the early stage of tryptophan (trp) catabolism along the kynurenine pathway (3). Several cell types such as macrophages, epithelial cells and dendritic cells (DCs) express IDO-1 that can be induced by proinflammatory cytokines, such as IFN-γ, TLR ligands, such as LPS, and interactions between immune cells through co-stimulatory molecules such as CD80 and CD86 (3-5). It is known that IDO-1 can affect immunity through two non-exclusive mechanisms: the establishment of a local response with "amino acid deprivation" that inhibits pathogen growth and the production of trp metabolites with immunomodulatory functions or cytotoxic agents that inhibit T cell activation and modulate the differentiation of naïve T cells into regulatory T cells (Tregs) (6, 7).

Our group has previously demonstrated a significant increase of IDO-1 in cells present in skin lesions of patients with multibacillary leprosy (lepromatous leprosy, LL) compared to patients with the paucibacillary form (tuberculoid leprosy, TT/BT) (8-10). Lipoproteins (19 kDa and 33 kDa) present in *M. leprae* plasma membrane are well known to activate monocytes and dendritic cells (DCs) through TLR2 (11). Analyses of skin lesions from leprosy patients show that TLR2 is strongly expressed in cells of paucibacillary patients, in contrast to poor expression in cells from multibacillary patient lesions (11). A subsequent study showed that activation of TLR2/1 leads to rapid differentiation of human peripheral monocytes in CD1b+ dendritic cells in paucibacillary patients and in DC-SIGN$^+$ cells in

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multibacillary patients (12), suggesting that TLR-induced monocyte differentiation in macrophages or dendritic cells influences the host response to *M. leprae* infection. Here, we investigated the ability of *M. leprae* and subcellular fractions to modulate IDO-1 expression and activity as well as their capacity to induce a tolerogenic or microbicidal phenotype in human monocyte-derived dendritic cells (mDCs).

Materials and Methods

Obtaining Buffy Coats

Buffy coats were obtained from healthy blood donors in the hemotherapy service of Clementino Fraga Filho University Hospital of the Federal University of Rio de Janeiro (UFRJ) through a technical-scientific partnership approved by the Research Ethics Committee of the Oswaldo Cruz Foundation (approval number: 1.538.467). Inclusion and exclusion criteria were the same as those used for screening in blood banks, and volunteers under 18 years of age whose serological screening was positive for Hepatitis B (HbsAg and Anti-HBc), Hepatitis C (HCV), AIDS (HIV-1/2 Ag + Ab combined test), Chagas Disease (Anti-*Trypanosoma cruzi*), Syphilis (VDRL - non-treponemal), HTLV-I and HTLV-II, Malaria and Cytomegalovirus (CMV) were excluded.

Obtaining human dendritic cells differentiated from monocytes (mDCs)

Human peripheral blood mononuclear cells (PBMCs) were obtained under endotoxin-free conditions by the Ficoll-Paque PLUS method (GE Healthcare). PBMCs were labeled with CD14 magnetic beads (20µl MicroBeads to 10^7 cells/MACS Miltenyi Biotec) for 15 minutes at 4°C and passed through a positive separation column. After monocyte separation, $CD14^+$ cells were cultured in the presence of medium containing IL-4 (10 ng/mL) and GM-CSF (50

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ng/mL) (PeproTech, NJ, USA) for 6 days at 37ºC with 5% CO_2. After this time the non-adherent cells (mDCs) were then counted and plated.

Cell culture stimuli and infection

mDCs were stimulated with 0.1, 1 or 10 µg/mL of irradiated *M. leprae,* and its membrane (MLMA) and soluble/cytosolic fractions (MLSA) (BEI Resources (NIH/ATCC), 200 µM of the IDO inhibitor 1-methyl-D-trytophan (1-MT, SigmaAldrich), 500 µg/mL of the anti-human TNF (Peprotech), 100 µg/mL of the anti-human IL-10 (Peprotech), 10 µg/mL of the TLR2 agonist Pam3Cys-Ser-(Lys)4 (SigmaAldrich) 100 µM of the inhibitory peptide for MyD88 homodimerization (IMGENEX) or 1 µg/mL of Mab-mTLR2 (InvivoGen), and incubated for 24 h at 37°C in 5% CO_2. *M. leprae* and its fractions were tested for purity and the absence of endotoxin. According to the limulus amebocyte lysate assay (Lonza, Basel, Switzerland), all stimuli used for *in vitro* cultures were shown to contain less than 0.1 U/mL endotoxin.

Flow cytometry

Panels of antibodies used for phenotypic detection and intracellular cytokine detection are described in Table 1. Following stimulation, $1x10^6$ mDCs were transferred from the plate to cytometry mini-tubes. Cells were washed and then fixed (2% paraformaldehyde, PFA). Subsequently, mDCs were permeabilized (0.15% saponin in PBS) and incubated for 30 minutes at 4°C with their respective antibodies. At the end of the incubation, cells were washed, suspended and cell phenotype was evaluated by flow cytometry (FACS Aria IIu). For each sample, a minimum of 10,000 events were acquired. The analysis was performed using the BD FlowJo software.

ELISA

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Supernatants were tested for the presence of tumor necrosis factor (TNF), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 (IL-12) and interleukin-15 (IL-15) cytokines using commercially available enzyme-linked immunosorbent assay (ELISA) (eBioscience) following the protocols supplied by the manufacturer.

High performance liquid chromatography analysis (HPLC)

Determination of IDO-1 activity in mDC culture supernatants was performed by determination of kynurenine and tryptophan levels (13) by HPLC. In an eppendorf tube, 165 µL of culture supernatant were homogenized with 5 µL tyrosine and 25 µL trichloroacetic acid (TCA). The tubes were then centrifuged for 10 minutes at 15,800 *xg*. After centrifugation, the supernatant was collected and 50 µL were injected into the reverse phase C18 column. Run detection was performed at 365 nm to detect kynurenine and 285 nm for tryptophan and the internal normalizer tyrosine. IDO activity was assessed by the ratio of kynurenine (kyn) to tryptophan (trp).

Co-culture of mDCs and lymphocytes

To assess the functional role of IDO-1, mDCs were obtained as described above and plated in 96-well plates ($2x10^4$). After the stimulus period, autologous lymphocytes were added at a ratio of 1 (mDC):20 (lymphocytes) for 5 days at 37ºC 5% CO_2. Subsequently, cells were harvested, washed and labeled with anti-FoxP3 Alexa Fluor 488/CD4 PE-Cy5/CD25 PE antibodies according to the specifications of the True-Nuclear Human Treg Flow ™ Kit (Biolegend). Cells were analyzed by flow cytometry (FACS Aria IIu).

Statistical analysis

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Analyses of the experiments were performed by Kruskall-Walis test or One-Way ANOVA. For all statistical analyses the value of $p \leq 0.05$ was considered significant. Statistical analyses were performed using the Windows GraphPad Prism version 8.0 software (GraphPad Software, San Diego, CA, USA).

Results

M. leprae increases IDO-1 expression and activity in mDCs

Previous data from our group have demonstrated that *M. leprae* induces the expression and activity of IDO-1 in human monocytes (8). In order to investigate whether *M. leprae* and its fractions are capable of modulating IDO-1 protein expression in mDCs, cells were stimulated with *M. leprae*, MLMA or MLSA for 24 hours. DCs were analyzed by flow cytometry to evaluate IDO-1 expression (**Figure 1A**) and a panel of specific markers was used to confirm their differentiation (**Supplementary Figure 1A-R**). *M. leprae* and MLMA fraction were efficient in inducing IDO-1 expression at 10 μg/mL, but not MLSA (**Figure 1B-C**). The kynurenine/tryptophan ratio in the supernatants reflects IDO-1 activity. To confirm if the enzymatic activity of IDO-1 was also modulated by different mycobacterial stimuli, the kynurenine/tryptophan ratio in the supernatants was analyzed by HPLC. As observed in **Figure 1D**, *M. leprae* (0.3 + 0.02 in ML *versus* 0.05 + 0.01 in NS, p = 0.01) and MLMA (0.24 + 0.01 in MLMA *versus* 0.05 + 0.01 in NS, p = 0.02) were able to significantly increase the enzymatic activity of IDO-1, but the same was not observed with the MLSA fraction. These data show that antigens present in membrane of the bacilli are able to induce IDO-1 activity.

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TNF is important in the induction of IDO-1 activity by *M. leprae* and its fractions in mDCs

Analysis of TNF^+ cells by flow cytometry revealed that the MLMA fraction was able to induce an increase in the frequency of double positive $IDO\text{-}1^+TNF^+$ cells when compared with non-stimulated cells (8.35 + 2.17 in MLMA *versus* 0.92 + 0.24 in NS, $p = 0.022$) (**Figure 2A-E**). However, neither *M. leprae* nor its fractions were able to induce an increase in the frequency of $IDO\text{-}1^+$ $IL\text{-}10^+$ cells (**Supplementary Figure 2**). Cytokine concentrations in the culture supernatants of mDCs stimulated with *M. leprae*, MLSA and MLMA for 24h were evaluated by ELISA. As observed in Figure 3, *M. leprae* was not able to significantly modulate the production of the cytokines tested. However, MLMA was able to induce a significant increase in TNF, IL-6 and IL-12 concentrations (**Figures 3A,C-D**) when compared to non-stimulated cells. In contrast, MLSA induced an increase in IL-10 levels (**Figure 3B**). There was no change in IL-15 production by mDCs after the stimuli with *M. leprae* and its fractions (not shown).

TNF, which is normally present in infectious environments, synergistically enhances IFN-γ-induced IDO-1 activity (14). Since the stimulation with MLMA induced an increase in TNF and stimulation with MLSA induced IL-10, we investigated the role of both IL-10 and TNF in the induction of IDO-1 expression and activity by *M. leprae* and its fractions on mDCs. As shown in Figure 4, the blockade of TNF in cultures stimulated with *M. leprae* or its fractions led to a significant decrease in IDO-1 activity in mDCs cultures, suggesting an important role of this cytokine in the induction of IDO-1 by *M. leprae* and its fractions. The same effect was not observed after IL-10 blockade (**Figure 4A-C**).

TLR2 is important for the induction of IDO-1 activity in mDCs

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M. leprae membrane contains lipoproteins that can activate TLR2 signaling triggering an inflammatory response (15-16) and therefore a role for this signaling pathway in IDO-1 induction in stimulated mDCs was investigated. As observed in Figure 5A, the blockade of TLR2 decreased IDO-1 activity induced by *M. leprae* and its fractions, demonstrating that IDO-1 activity and expression in *M. leprae*-stimulated mDCs is TLR2-dependent. The TLR2 agonist Pam3Cys was used as positive control, suggesting that TLR2 pathway is involved in IDO 1 induction in mDCs (0.25 + 0.01 in ML *versus* 0.03 + 0.01 in ML + TLR2, p = 0.20; 0.20 + 0.02 in MLMA *versus* 0.01 + 0.002 in MLMA + TLR2, p = 0.01; 0.13 + 0.03 in MLSA *versus* 0.02 + 0.006 in MLSA + TLR2, p = 0.01). In addition, we used a peptide inhibitor of the MyD88 pathway in parallel to a peptide control of the same pathway. A decrease in IDO-1 activity was observed when the inhibitory peptide was added to the cultures (0.22 + 0.03 in ML *versus* 0.02 + 0.005 in ML + αMyD88, p = 0.01; 0.18 + 0.02 in MLMA *versus* 0.05 + 0.005 in MLMA + αMyD88, p = 0.007; 0.08 + 0.02 in MLSA *versus* 0.01 + 0.007 in MLSA + αMyD88, p = 0.03) (**Figure 5B**).

***M. leprae* and its fractions influence the tolerogenic profile of lymphocytes**

In order to verify whether the increase in IDO-1 expression and activity influenced the lymphocyte phenotype after priming by the mDCs, a co-culture assay was performed in which the mDCs stimulated with *M. leprae* and its fractions were cultivated with autologous lymphocytes in a proportion of 20 lymphocytes:1 mDC.

Through flow cytometric analysis (**Supplementary Figure 3 and Figure 6A-H**), an overall increase in Treg frequencies in MLSA or ML-stimulated mDCs co-cultures was observed. Inhibition of IDO-1 activity significantly abrogated Treg differentiation for those stimuli, suggesting that acquisition of the regulatory profile during co-cultures may be IDO-1

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dependent (9.19 + 2.20 in ML *versus* 0.24 + 0.10 in ML + 1MT, p=0.007 and 7.32 + 0.73 in MLSA *versus* 0.24 + 0.10 in MLSA + 1MT, p = 0.007) **(Figure 6I)**.

Discussion

The characterization of infectious environments is important to define how innate immunity influences the subsequent development of adaptive immunity. Regulatory mechanisms that operate at both stages of immunity are also critical in determining how the immune response will dictate the severity of an infectious process. In this context, we have explored the regulatory mechanisms exerted by the enzyme IDO-1 during *M. leprae* infection. At the lepromatous leprosy pole, high IDO-1 expression along with high levels of IL-10 and TGF-β could be responsible for the pronounced reduction of the antigen-specific cellular immune response observed in these patients (2, 8). At the tuberculoid pole of the disease, where the expression of IFN-γ predominates, IDO-1 would be induced mainly by this cytokine (10) and could be associated with microbicidal activity, suggesting that in leprosy IDO-1 may have a dual role, being tolerogenic or microbicidal depending on the environment and the cytokines involved in its induction.

In skin lesions of leprosy patients, in addition to intact bacilli, some mycobacterial fractions from the membrane and the *M. leprae* cytosol exposed after bacterial death, which may be a result of both the host immune response and the treatment, should induce immune responses (1). Thus, in addition to the use of irradiated *M. leprae*, mDCs were stimulated by the membrane fraction of *M. leprae* (MLMA) and the soluble fraction of *M. leprae* (MLSA), mimicking the tissue microenvironment.

We are the first to demonstrate that *M. leprae* is able to induce IDO-1 activity in mDCs when compared to unstimulated cells. In parallel, the intracellular expression of IDO-1

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was analyzed by flow cytometry and we observed that *M. leprae* and its fractions were able to increase IDO-1 expression, with a higher increase after MLMA stimulation, but not MLSA. It has already been shown that *M. tuberculosis* infection leads to upregulation of IDO-1 expression in murine macrophages (17). In addition, elevated IDO-1 activity was described in many chronic inflammatory syndromes, including cancer, infections, allergies, and pregnancy (18).

The production of cytokines in these environments was evaluated. It should be noted that among the stimuli studied, MLMA significantly induced an increase in the production of inflammatory cytokines, such as TNF, IL-6 and IL-12. It was also observed that the MLMA fraction induced an increase in the frequency of $IDO^{+}TNF^{+}$ cells. TNF has been suggested to be one of the most important cytokines for the induction of IDO-1. The contribution of TNF to the activation of IDO-1 induced by LPS has already been observed (19). In addition, TNF mediates stress-induced depression by positively regulating the IDO-1 enzyme (20). As TNF modulates the response to cytotoxicity and the production of inflammatory cytokines (21), our data suggest that the MLMA fraction may be involved in a microbicidal effect.

Our results showed that *M. leprae* and MLSA led to an increase in IL-10 secretion, which could suggest a regulatory effect, since an earlier study (2) demonstrated that *M. leprae* is able to induce the gene and protein expression of IDO-1 in human monocytes by an IL-10-dependent mechanism. In leprosy, IL-15 induces the vitamin D-dependent antimicrobial pathway and this ability is consistent with the expression of this cytokine in self-limited T-lep lesions (22). Here, we investigated the secretion of this cytokine in cell supernatants and observed that there was no change between the stimuli used (not shown).

Our data also show that blocking TNF decreases IDO-1 activity in mDCs by *M. leprae* and its fractions, unlike IL-10 blockade. These results led us to hypothesize that TNF

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is induced at different levels by different antigens present in cytosol and on bacilli cell membrane and that this cytokine is important for IDO-1 expression and activity in human mDCs. In contrast, IL-10 is not as efficient in inducing IDO-1 expression and activity in mDCs as observed previously in human monocytes stimulated with *M. leprae* (2).

Recognition of components of the mycobacterial cell wall, such as sugars, lipids and peptides can induce macrophages to secrete TNF and other cytokines like IL-12 that are essential for the development of an inflammatory response (23). TLR2, activated by lipoarabinomannan (LAM), is required for the induction of TNF in macrophages by *M. tuberculosis*. Previous studies have further shown that the expression of IDO-1 in DCs can be induced by inflammatory cytokines and TLR ligands (6).

Lipoproteins from *M. tuberculosis*, such as 19kD and mannose-capped lipoarabinomannan (ManLAM), are TLR2 ligands that trigger the development of regulatory immune responses (24-26). Previous studies have shown that Lisophosphatidylcholine (LPC) subverts TLR-mediated signaling in DCs, which drives cell differentiation to a tolerogenic phenotype (27). In addition, it was demonstrated that tolerogenic effects induced by IDO-1 depend on TLR2/6-induced JNK signaling by *Faecalibacterium prausnitzii* and ectonucleotidase activity (28). Our data demonstrated that *M. leprae* induces an increase in IDO-1 expression and activity in mDCs, and that the membrane components of the bacilli play an important role in this context via TLR2 signaling associated with the production of inflammatory cytokines. We cannot exclude the involvement of TLR4 on IDO-1 activation induced by *M. leprae*. Although *M. leprae* is an acid-fast intracellular Gram-positive bacillus, there are evidences that it can activates TLR4 (29). Mycobacterial glycolipids such as the lipooligosaccharides (LOS) contain the lipid A portion, which is responsible for the LPS interaction with TLR4.

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IDO-1 may require TLR-MyD88-NF-κB signaling to promote the development of colitis (30). Since NF-κB activation depends on MyD88 we performed assays using a MyD88 inhibitor, and we observed that the absence of MyD88 in cultures of mDCs leads to a decrease in the activity of IDO-1 also suggesting it might be the case.

A key mechanism involved in mDCs-mediated immunosuppression is the expression of IDO-1 (31). The interaction of DCs with TLR2 and TLR4 treated with ES L1 (excretory-secretory muscle larvae) promotes the expansion of $CD4^{+}$ $CD25^{high}Foxp3^{high}$ producing IL-10 and TGF-β cells in an IDO-1 dependent form (32). 6-formylindolo[3,2-b]carbazole (FICZ)-treated DCs are able to induce differentiation of naive T lymphocytes into regulatory-like T cells (33). Our co-culture data showed that *M. leprae* is capable of enhancing the expression of regulatory T $CD4^{+}CD25^{+}FOXP3^{+}$ lymphocytes (Treg) by an IDO-1 dependent form. MLSA, which increased IL-10 secretion, is capable of enhancing the expression of Treg by an IDO-1 independent form. It has also been shown that lepromatous patients increase the expression of regulatory T cells, one factor that may explain the large number of bacilli in these individuals (34). These findings provide a new insight into the mechanisms of TLR-induced tolerogenic phenotype in DCs, which may help to better understand the processes involved in the induction and resolution of chronic inflammation and tolerance.

Together, *M. leprae*-induced IDO-1 axis may play an important role in controlling the immunity and severity of leprosy. In clinical terms, our hypothesis is that in multibacillary patients, the high flow of living and dead bacilli induces IDO-1 by an IL-10-dependent mechanism that in turn leads to a tolerogenic effect, decreasing the cellular immune response. In paucibacillary patients and in the reaction episodes, the exposure of bacilli membrane antigens favors a context similar to that described by mDCs stimulation with MLMA, that is, the increase of IDO-1 due to the activation of TNF and IL-12, which consequently leads to an

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increase in the pro-inflammatory response and could contribute to a decrease in bacillary load.

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Legends

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Figure 1

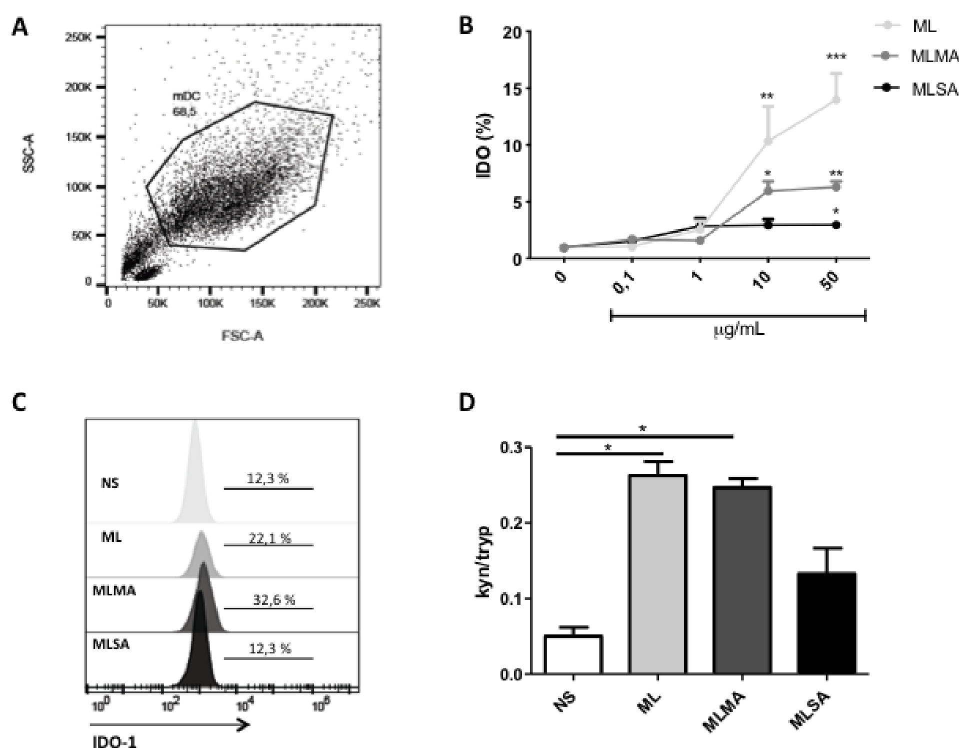

Figure 1: IDO-1 activity and expression in mDCs. The differentiated mDCs were plated ($1x10^6$ cells per well). (A) Gate strategy for flow cytometry analysis. (B) mDCs were stimulated with *M. leprae* (ML), membrane fraction of *M. leprae* (MLMA) or soluble fraction of *M. leprae* (MLSA) at 0.1, 1, 10 or 50 µg/mL, for 24 hours. After this time, cells were labeled with anti-IDO-1 intracellular antibody. Acquisition was performed in a BD FACS Aria IIu flow cytometer and analysis performed using FlowJo software. NS - unstimulated cell. The graph shows the percentage of IDO-1-positive mDC of three independent experiments performed in triplicate. (C) Representative histograms of mDCs stimulated with ML or its fractions. (D) Supernatants from cell cultures were collected and IDO-1 activity was assessed by the kynurenine/tryptophan ratio (kyn/trp) by HPLC. The plot represents the mean ± standard deviation of three independently performed experiments. * $p < 0.05$; ** $p<0.01$; *** $p<0.001$.

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Figure 2

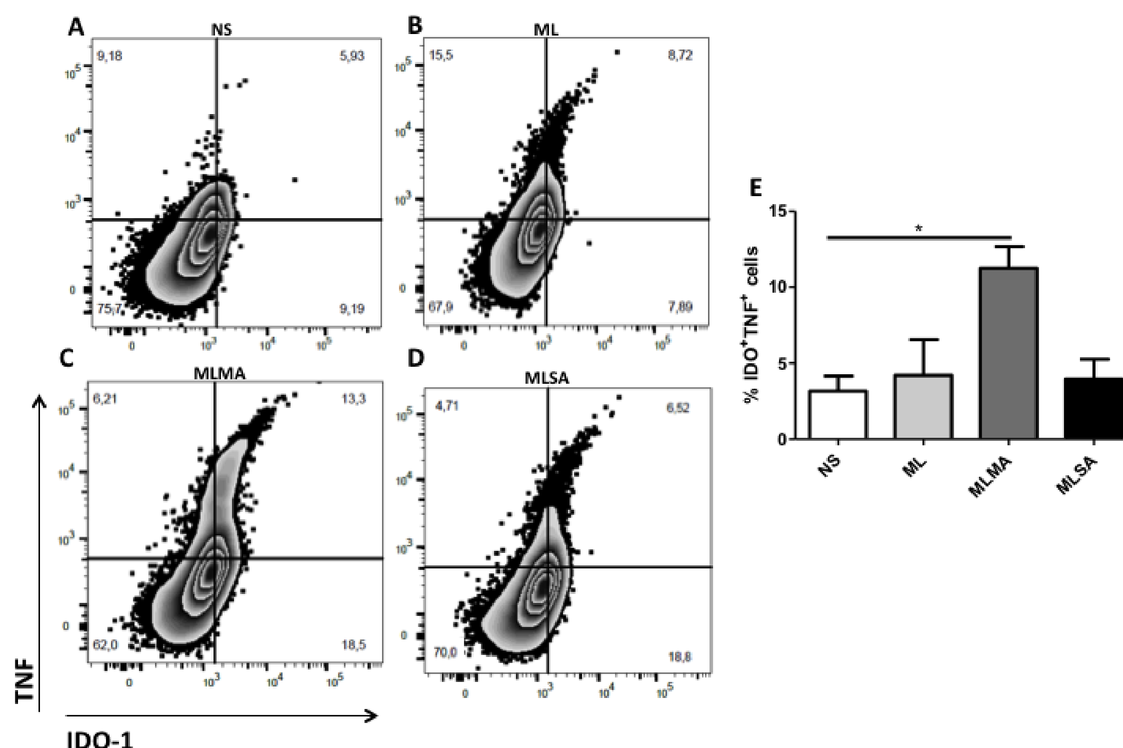

Figure 2: MLMA is able to increase the frequency of IDO-1^{+}TNF^{+} mDCs. DCs differentiated from monocytes were plated (1x10^{6} cells per well) and stimulated with *M. leprae* (ML), membrane fraction of *M. leprae* (MLMA) and soluble fraction of *M. leprae* (MLSA) at 10 µg/mL, for 24 h, or not (NS - unstimulated cell). Cells were labeled with anti-IDO-1 PE and Alexa Fluor 700 TNF intracellular antibodies. Acquisition was performed in a BD FACS Aria IIu flow cytometer and analysis performed using FlowJo software. Analysis is representative of cells from a donor from 4 experiments. For each sample, a minimum of 10,000 events were analyzed. * $p < 0.05$ (Mann-Whitney).

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Figure 3

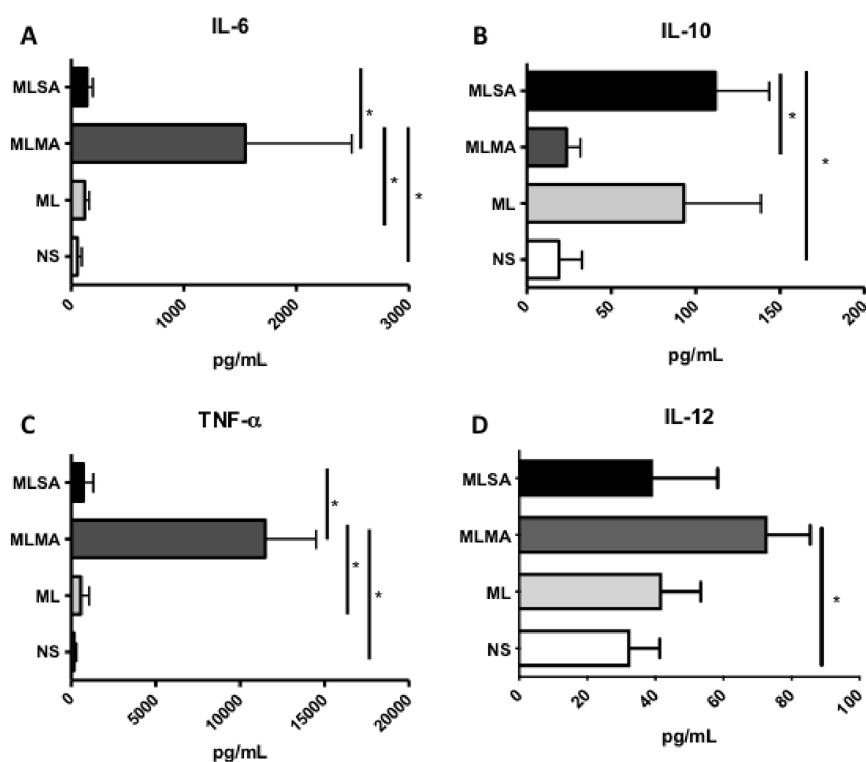

Figure 3: Differential cytokine production in cell supernatants from MLMA or MLSA-stimulated mDCs. mDCs were plated (1x10^{6} cells per well) and stimulated with *M. leprae* (ML), membrane fraction of *M. leprae* (MLMA) and soluble fraction of *M. leprae* (MLSA) at 10 µg/mL, for 24 h, or not (NS - unstimulated cell). (A-D) Supernatants from these cultures were then collected and TNF, IL-6, IL-10 and IL-12 concentrations were evaluated by ELISA. Graphs represent the mean ± standard deviation of four independently performed experiments. * $p < 0.05$ (One-Way ANOVA).

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Figure 4

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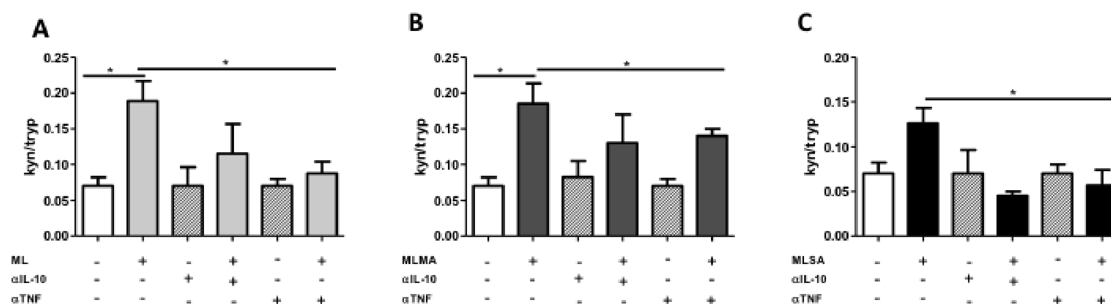

Figure 4: Importance of TNF in the induction of IDO-1 activity in *M. leprae*-stimulated mDCs. mDCs were plated (1x10^6 cells per well) and 1 hr prior to challenge, anti-human IL-10 (100 µg/ml) or anti-human TNF (500 µg/mL) neutralizing antibody were added at cultures followed by stimuli: *M. leprae* (ML), membrane fraction of *M. leprae* (MLMA) or soluble fraction of *M. leprae* (MLSA) were added 10 µg/mL or not (NS - non-stimulated cell). After 24h, supernatants were collected and IDO-1 activity was assessed by the kynurenine/tryptophan ratio (kyn/trp) by HPLC. The plot represents the mean ± standard deviation of four independently performed experiments. * p <0.05 (Kruskall-Wallis).

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Figure 5

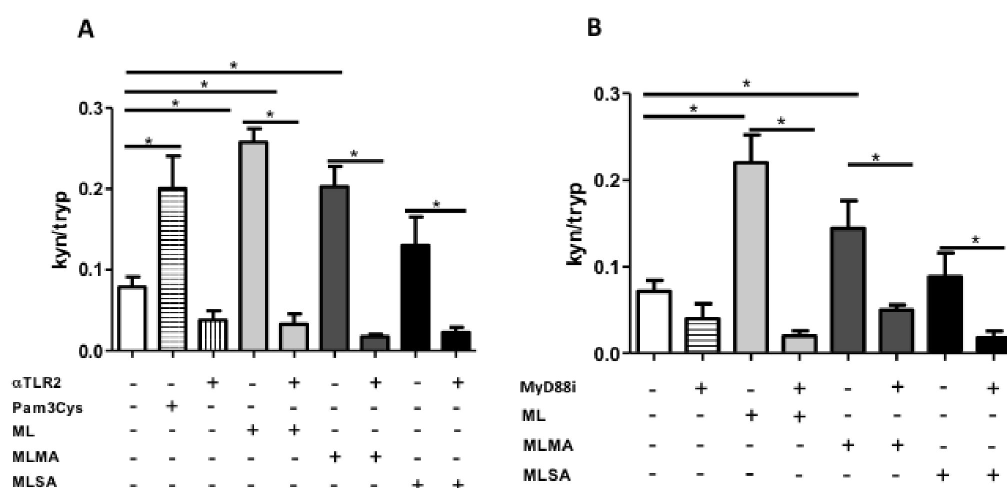

Figure 5: TLR2 is important in inducing IDO-1 activity in mDCs. mDCs were plated ($1x10^6$ cells per well) and 1 hr prior to challenge, anti-human TLR2 (1 µg/mL) neutralizing antibody or MyD88 peptide inhibitor (MyD88i; IMGENEX, San Diego, CA) (100 µM) was added, followed by stimuli: *M. leprae* (ML), membrane fraction (MLMA) or soluble fraction (MLSA) were added at 10 µg/mL, TLR2 agonist Pam3Cys was used as positive control (10 µg/mL) for 24 h at 37ºC 5%CO_2. Controls are non-stimulated cultures. Supernatants from these cultures were collected and IDO-1 activity was assessed by the kynurenine/tryptophan ratio (kyn/trp) by HPLC. The plot represents the mean ± standard deviation of five independently performed experiments. *$p < 0.05$ (Mann-Whitney).

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Figure 6

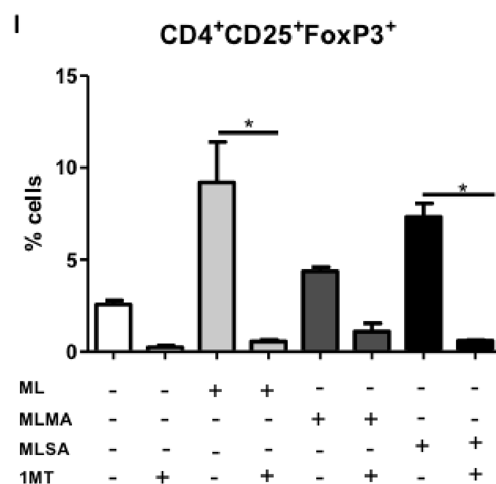

Figure 6

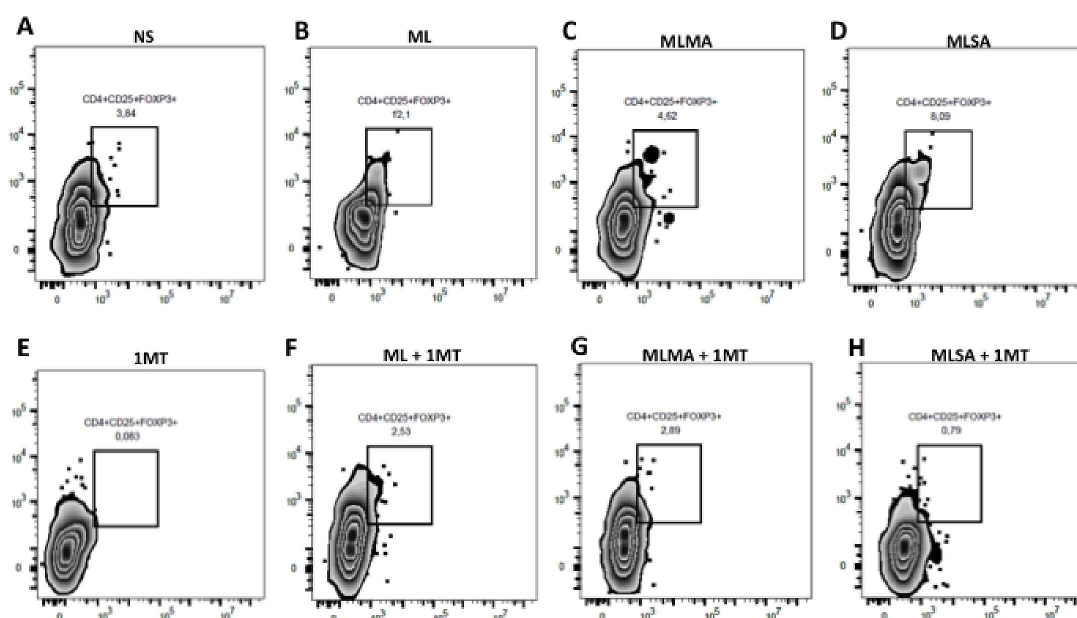

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Figure 6: Increased Treg expression by *M. leprae* and MLSA. mDCs were plated ($1x10^4$ cells per well) and stimulated with *M. leprae* (ML), membrane fraction of *M. leprae* (MLMA) and soluble fraction of *M. leprae* (MLSA) at 10 µg/mL, for 24 h, or 1MT (IDO inhibitor - 1 hour before other stimuli) or not (NS - unstimulated cell). Cells were washed and the autologous lymphocytes (20 Lymphocytes:1 mDC) were cultured for 5 days. Cells were then labeled with intracellular anti-CD4, CD25 and FoxP3 or isotype antibodies. Acquisition was performed by BD FACS Aria IIu flow cytometry and analysis performed using FlowJo software. Analysis is representative of cells from a donor from 4 experiments. For each sample, a minimum of 10,000 events were analyzed. *p <0.05 (Mann-Whitney).

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Table 1. Antibodies used in flow cytometry.

Antibody	Clone	Catalog number	Company	Fluorochrome
CD14	61D3	120149-42	eBioscience	PE
HLA-DR	G46-6	555811	BD eBioscience	FITC
CD11c	3.9	301606	BioLegend	PE
CD209	eB-h209	11-209973	eBioscience	FITC
CD86	IT2.2	15-0869-71	eBioscience	PE-Cy5
CD83	HB1Se	305320	BioLegend	PerCP-Cy5.5
CD304	AD5-17F6	130-090-900	Milteny Biotec	APC
CD123	6H6	306012	BioLegend	APC
IDO	700838	IC6030P	R&D	PE
TNF-α	MAb11	561023	BD eBioscience	AlexaFluor700
IL-10	127107	IC2172A	R&D	APC

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