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The Effect of Apoptotic Cell Recognition on Macrophage Polarization and Mycobacterial Persistence

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Running Head: Efferocytosis in *Mycobacterium leprae* pathogenesis

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Abstract

Intracellular *Mycobacterium leprae* infection modifies host macrophage programming, creating a protective niche for bacterial survival. The milieu regulating cellular apoptosis in the tissue plays an important role in defining susceptible and/or resistant phenotypes. A higher density of apoptotic cells has been demonstrated in paucibacillary leprosy lesions than in multibacillary ones. However, the effect of apoptotic cell removal on *M. leprae*-stimulated cells has yet to be fully elucidated. In this study, we investigate whether apoptotic cell removal (efferocytosis) induces different phenotypes in pro- (Mϕ1) and anti- (Mϕ2) inflammatory macrophages in the presence of *M. leprae*. We stimulated Mϕ1 and Mϕ2 cells with *M. leprae* in the presence or absence of apoptotic cells and subsequently evaluated the *M. leprae* uptake, cell phenotype, and cytokine pattern in the supernatants. In the presence of *M. leprae* and apoptotic cells, Mϕ1 macrophages changed their phenotype towards Mϕ2, displaying increased CD163 and SRA-I expression as well as higher phagocytic capacity. Efferocytosis increased *M. leprae* survival in Mϕ1 cells, accompanied by reduced IL-15 and IL-6 and increased TGF-β and IL-10 secretion. Mϕ1 cells primed with *M. leprae* in the presence of apoptotic cells induced the secretion of Th2 cytokines IL-4 and IL-13 in autologous T cells when compared with cultures stimulated with *M. leprae* or apoptotic cells alone. Efferocytosis did not alter the Mϕ2 cell phenotype or cytokine secretion profile, except for TGF-β. Based on these data, we suggest that, in paucibacillary leprosy patients, efferocytosis contributes to mycobacterial persistence by increasing the Mϕ2 population and sustaining the infection.

Introduction

Macrophages have remarkable plasticity, allowing them to efficiently respond to environmental signals and change their phenotype. Their physiology can be markedly altered by both innate and adaptive immune responses (1-8). Pro-inflammatory (Mϕ1) and anti-inflammatory (Mϕ2) macrophage polarization contribute to the resolution of inflammatory processes. The presence of the Mϕ2 macrophage population is important for maintaining a basal anti-inflammatory environment in tissues continuously exposed to exogenous agents such as skin. Both granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) contribute to macrophage and dendritic cell development (9-12) but influence the macrophage polarization state in an opposite manner. Whereas Mϕ1 polarized in the presence of GM-CSF promotes type 1 immunity, Mϕ2 polarized with M-CSF subverts type 1 immunity and thus may promote immune escape and chronic infection (13).

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*, an obligate intracellular pathogen. The disease is characterized by a spectrum with two polar clinical forms. Tuberculoid or paucibacillary leprosy is characterized by a robust Th1 immune response, strong cellular immunity, low bacillary counts, and small lesion numbers. On the other hand, lepromatous or multibacillary leprosy feature high levels of Th2-type cytokines, a high bacillary load, and many skin lesions (14-17).

Previous studies by our group demonstrated that *M. leprae* can lead to macrophage apoptosis through a mechanism involving the expression of TNF and the proteasome function (18-20). In addition, in comparing lesions from multi- and paucibacillary patients, Walsh and colleagues reported that apoptosis was more frequent in paucibacillary lesions, suggesting that the activation of apoptosis could act as a containment mechanism of bacilli multiplication and spread (21).

Macrophages undergo dramatic molecular and functional changes upon encounter, interaction with, and uptake of apoptotic cells during inflammation resolution. We herein demonstrated that, in the presence of *M. leprae*, the clearance of apoptotic cells (efferocytosis) induces pro-inflammatory macrophage deviation towards an anti-inflammatory phenotype. Although efferocytosis has been described as an antimicrobial effector mechanism during *M. tuberculosis* infection (22-23), our findings suggest that, in leprosy, efferocytosis may explain the persistence of mycobacterial disease in paucibacillary patients regardless the capacity of these patients to mount a cellular immune response by modulating the macrophage phenotype and function in cell lesions.

Materials and Methods

Patients and clinical specimens

The acquisition of all specimens was approved by the Oswaldo Cruz Foundation Human Ethics Committee, Rio de Janeiro, RJ, Brazil. Leprosy patients were classified according to the Ridley and Jopling classification scale (24).

Buffy coats were obtained from normal donors (healthy controls: HCs) at the Hemotherapy Service of the Clementino Fraga Filho University Hospital, associated with the Federal University of Rio de Janeiro, RJ, Brazil, in accordance with the guidelines set down in the Declaration of Helsinki.

Immunohistochemical studies

Leprosy patient skin biopsies (5 BT, 5 LL) were obtained at diagnosis and prior to treatment. For routine histopathological analyses, all skin tissues were stained with haematoxylin and eosin (H&E) in addition to the Wade method. To detect arginase, immunoperoxidase labeling of cryostat sections was performed. The cryostat sections were fixed in acetone, hydrated in $Ca^{+2}Mg^{+2}$-free phosphate-buffered saline (PBS) 0.01M, and incubated with hydrogen peroxide 0.3% in PBS for 10 min to quench endogenous peroxidase activity. Unspecific binding sites were blocked with horse normal serum (Kit ABC Elite, Vector Laboratories, Burlingame, CA, USA). The mouse anti-human antibody against arginase (1:50, BD Biosciences, San Jose, CA, USA) was diluted in PBS 0.01M and incubated for 1h at room temperature. The sections were washed three times and incubated with biotinylated horse anti-mouse immunoglobulins (Kit ABC Elite, Vector Laboratories,) for 1h at room temperature. After washing, the sections were incubated for 40 min with avidin-biotin complex (Kit ABC Elite, Vector Laboratories) for signal amplification. The reaction was developed at room temperature

in a solution of 3-amino-9-ethylcarbazole for 10 min (AEC Peroxidase Substrate Kit, Vector Laboratories). Slides were counterstained with Mayer's haematoxylin and mounted with aqueous mount medium Faramount (Dako, Thousand Oaks, CA, USA). Images were obtained via a Nikon Eclipse microscope with Infinite Capture software (Lumenera Corporation, Ottawa, ON, Canada).

Cell culture

Human peripheral blood mononuclear cells (PBMC) were isolated under endotoxin-free conditions from buffy coats by the Ficoll-Hypaque method (Pharmacia Fine Chemicals, Piscataway, NJ, USA). Monocytes were purified from PBMC by magnetic cell sorting using CD14 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). GM-CSF and M-CSF promote the acquisition of distinct morphology, pathogen susceptibility, and inflammatory functions in macrophages (13, 25-28). Therefore, although they are used interchangeably for the *in vitro* generation of human monocyte-derived macrophages (29), GM-CSF- and M-CSF-polarized macrophages are considered pro- and anti-inflammatory macrophages, respectively (13) and, by analogy to widely-accepted nomenclature (''classical''/Mϕ1 and ''alternative''/Mϕ2 macrophage polarization states), will hereafter be referred to as Mϕ1 (differentiated with GM-CSF) and Mϕ2 (differentiated with M-CSF) macrophages. To generate Mϕ1 and Mϕ2 cells, monocytes were resuspended in RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2mM L-glutamine, and 10% fetal calf serum (FCS, Gibco BRL, Gaithersburg, MD, USA) containing 50 ng/mL of GM-CSF (R&D Systems, Minneapolis, MN, USA) or 50 ng/mL of M-CSF (R&D Systems), respectively and cultured for 6 days in 6- or 24-well plates (Costar, Cambridge, MA, USA) at 1 x 10^6 cells/mL.

Cell Stimulation and Infection

Irradiated armadillo-derived *M. leprae* whole cells probe-sonicated with a Sanyo sonicator to >95% breakage (NIH/NIAID "Leprosy Research Support" from Colorado State University) were added to the Mϕ1 and Mϕ2 cultures at 10 µg/mL. When necessary, apoptotic cells were added to the culture after 30 min; and cells were incubated for 90 minutes or 24 hours. Live *M. leprae* Thai-53 purified from hind footpads of BALB/c athymic nude mice was obtained from the National Hansen's Disease Program and the Lauro de Souza Lima Institute (Bauru, São Paulo, Brazil) and added to the cultures at a MOI of 10 for 24h in the presence or absence of apoptotic cells. Bacteria were counted by acid-fast staining (BBL TB Ziehl-Neelsen Stain Kit, BD Biosciences). Mycobacteria were PKH67 Green labeled according to the manufacturer's instructions (PKH67 Green Fluorescent Cell Linker Kit for General Cell Membrane Labeling, Sigma Aldrich, Saint Louis, MO, USA). Mycobacterial viability was determined by the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Life Technologies, Carlsbad, CA, USA), as previously described (30). In arginase inhibitory studies, Mϕ1 cells were pre-treated for 30 min with 10 µM N^{ω}-hydroxy-nor-arginine (nor-NOHA, Cayman Chemical, Ann Arbor, MI, USA) or vehicle (0.01% dimethyl sulfoxide) before stimulation with *M. leprae* and apoptotic cells.

Induction of apoptosis

Jurkat T cells were exposed to ultraviolet (UV) irradiation (254 nm) for 10 minutes and cultured in RPMI 1640 without serum for 3h in 5% CO_2 at 37°C. Human neutrophils were separated by Dextran sedimentation, followed by discontinuous isotonic Percoll gradient centrifugation. Human neutrophils (>95% pure) were cultured at 37°C and 5% CO_2 atmosphere at a concentration of 5×10^6/mL in DMEM with 10% autologous

serum for 20h to undergo apoptosis. Apoptosis of these cells was confirmed by light microscopy and Annexin V and propidium iodide staining (PI; BD Pharmingen, San Jose, CA, USA). Apoptotic Jurkat T cells (ApoJ) and apoptotic neutrophil (ApoN) were used when approximately 80% apoptotic cells were obtained (Annexin V positive, PI negative). When necessary, a PKH 26 Red Fluorescence cell linker Kit (Sigma-Aldrich) was used to label Jurkat cells for 2 min. Reaction was halted with 10% FCS (Gibco BRL); and cells were washed twice in RPMI and then resuspended in PBS.

Apoptosis assay

Phosphatidylserine (PS) externalization, an early-stage apoptotic event, was assessed by the binding of fluorescently-labelled Annexin V (FITC). Late-stage apoptosis and necrosis were measured by simultaneous staining with PI (propidium iodide) using the BD Pharmingen™ Annexin V-FITC apoptotic detection kit according to the manufacturer's instructions. Cells were harvested by centrifugation (2500 *xg* at 4°C for 5 min) after an ice-cold bath and washed three times in chilled PBS. Pellets were resuspended in 500 µL of 1X Binding Buffer (0.01 M Hepes, 0.14 M NaCl, and 2.5 mM $CaCl_2$, pH 7.4). A 100 µL fraction of the cell suspension was aliquoted into flow cytometry tubes and 5 µL of both PI and Annexin V-FITC were added. The tubes were then briefly vortex mixed. The cell suspension was incubated for 15 min at room temperature (22°C) in the dark. The percentage of cells undergoing early-stage apoptosis (Annexin V-FITC positive) and late-stage apoptosis/necrosis (Annexin V-FITC and PI positive) were measured with excitation at 488 nm and emission in FL1 (525 nm) for FITC and excitation of 536 nm and emission in FL3 (610 nm) for PI in an Accuri flow cytometer (BD Biosciences). Data were collected using CFlow software, and 10,000 events were analyzed per sample.

177 ***Evaluation of Mycobacterium leprae uptake***

178 Prior to bacterial interaction assays, *M. leprae* was stained via the PKH 67 Green
179 Fluorescence cell linker Kit (Sigma-Aldrich) according to the manufacturer's
180 instructions. Mϕ1 and Mϕ2 cells were stimulated with PKH 67-labeled *M. leprae* (10
181 µg/mL); and 2h or 24h post-infection, the index of bacterial association was determined
182 by flow cytometry and expressed as a percentage of PKH67-*M. leprae*$^{+}$ cells. To
183 determine bacterial uptake, the fluorescent signal of extracellular bacteria was quenched
184 with trypan blue after incubation time. The internalization of *M. leprae* was evaluated
185 by flow cytometry, as previously described (31).

186 ***FACS analysis of macrophage phenotypes***

187 To analyze the expression of the scavenger receptors CD163 and SRA-I, Mϕ1 and Mϕ2
188 macrophages were collected with a cell scraper after 24h of culture. Cells were stained
189 for 30 min at 4°C with 1:50 APC-conjugated anti-CD163 monoclonal antibody and 1:50
190 PE-conjugated anti-SRA-I monoclonal antibody (R&D Systems). Gates were defined
191 for collection and 20,000 live events were analyzed on a C6 Accuri cytometry using
192 Cflow software (BD Biosciences).

193 ***Cytokine detection by enzyme-linked immunosorbent assay (ELISA)***

194 Supernatants from Mϕ1 and Mϕ2 cells were tested for the presence of cytokines and
195 growth factors using commercially available ELISA Kits for IL-6, IL-10, IL-15, IFN-γ
196 and TGF-β (eBioscience, San Diego, CA, USA) following the protocols supplied by the
197 manufacturers.

198

Ultrastructural analysis

Macrophage ultrastructure was evaluated after stimulation with *M. leprae* (10 µg/mL) in the presence or absence of apoptotic cells for 90 min at 37°C. Cells were washed with PBS and fixed with glutaraldehyde 2.5% in sodium cacodylate buffer 0.1M, pH 7.2, and 3.5% sucrose for 1h at 4°C. Cells were then washed in the same buffer and fixed with 1% Osmium Tetroxide (OsO_4) for 1h at 4°C. Cells were washed in cacodylate buffer, dehydrated in serially-concentrated acetone (30, 50, 70, 90, and 100%), infiltrated with a mixture of 100% acetone and resin PolyBed 812, and polymerized at 60°C for 2 days. After polymerization, ultrafine sections were made (Reichert ultramicrotome OmU3) and collected on copper grids of 300 mesh, contrasted with 5% uranyl acetate and citrate lead, and observed under a Jeol JEM-1011 transmission electronic microscope.

Molecular determination of **M. leprae** *viability*

The viability of *M. leprae* was measured as previously described (32) with some modifications. Briefly, *M. leprae* RNA and DNA were simultaneously extracted by the TRIzol method (Life Technologies) according to the manufacturer's recommendations through single-tube homogenization using the Fast Prep FP 24 instrument (MP Biomedicals, Santa Ana, California, USA). Prior to reverse transcription, DNA was removed from the RNA preparations using the DNA-free Turbo kit (Ambion, Life Technologies); and RNA was reverse transcribed using random primer and SuperScript III following the manufacturer's instructions (Invitrogen, Life Technologies). *M. leprae* viability was estimated from the levels of 16S rRNA normalized against measured 16S rDNA using a TaqMan-based real-time PCR assay, as described (32).

Real time PCR

TaqMan PCR was performed via the universal PCR Master Mix (×2) and specific primer and probes (Applied Biosystems, Life Technologies). PCR was performed in the ABI Prism 7000-sequence detection system (Applied Biosystems) at 50°C for 5 min, 95°C for 10 min, 50 cycles of 95°C for 15s, and 60°C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. Arginase 1 mRNA was quantified using the $2^{-\Delta\Delta Ct}$ method for the PBMC samples.

Lymphocytic stimulation test

$CD3^+$ T cells were isolated from PBMCs with magnetic microbeads (Miltenyi Biotec). Lymphocytes were incubated at a ratio of 1 Mϕ1: 10 T cells. Mϕ1 cells were previously stimulated with *M. leprae* (10 μg/mL) for 24h in the presence or absence of apoptotic cells. Cells were co-cultured for 48 hours, and supernatants were harvested and stored until cytokines were analyzed by ELISA.

Statistical analysis

Results were reported as pooled data from the entire series of experiments. GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was used for all analyses; and samples were analyzed by ANOVA with a Tukey's post test. A $p < 0.05$ was deemed to be significant.

Results

M. leprae stimulation did not change the phenotype of differentiated macrophages

Mϕ1 and Mϕ2 cells were obtained by differentiation of purified human $CD14^+$ monocytes in the presence of GM-CSF or M-CSF, respectively, as previously described (13,33). It was observed that after 6 days of culture, the majority of Mϕ1 cells displayed a classical adherent "fried egg" morphology (Fig. 1A). On the other hand, Mϕ2 primarily appeared as adherent cells with a stretched, spindle-like morphology (Fig. 1A). Previous results of our group have demonstrated that *M. leprae* is able to induce apoptosis in human monocytes by a mechanism that involves TNF, and, although necessary, *M. leprae* phagocytosis is not crucial for cell death (18). Thus, we tested whether *M. leprae* was able to induce cell death in macrophages differentiated *in vitro*. *M. leprae* did not affect the cell viability of Mϕ2 macrophages. However, in Mϕ1 cells, *M. leprae* increased the percentage of apoptotic cells (Annexin V^+PI^-) when used at 20 µg/mL (Fig. S1). Interestingly, *M. leprae* was not able to induce apoptosis either in Mϕ1 or Mϕ2 when used at 10 µg/mL. Since our main interest was to investigate the role of efferocytosis in the context of the immune response against *M. leprae*, we chose to use *M. leprae* at 10 µg/mL in all experiments performed in this study to avoid any influence of Mϕ1 or Mϕ2 apoptosis in the observed immune response.

Analysis of the Mϕ2 phenotypic markers CD163 and SRA-I revealed that M-CSF-differentiated cells exhibited higher levels of these molecules when compared to GM-CSF-differentiated cells (CD163: Mϕ1 = 1.35 ± 0.46% *vs.* Mϕ2 = 6.57 ± 1.13%, $p<0.05$; SRA-I: Mϕ1 = 2.57 ± 0.41 *vs.* Mϕ2 = 6.83 ± 2.33, $p<0.05$). Nevertheless, *M. leprae* stimulation did not alter CD163 or SRA-I expression in either type of macrophage (Fig. 1B-C).

Since previous work has demonstrated differential regulation of macrophage functional programs by IL-10 and IL-15 (34), we investigated whether macrophages polarized *in vitro* could be better characterized by IL-10 and IL-15 production. We found that Mϕ2 secreted less IL-15 while producing higher IL-10 levels. Our results showed that Mϕ1 produced approximately 2.7 times more IL-15 (473.1 ± 55.1 pg/mL) than Mϕ2 macrophages (175.8 ± 9.8 pg/mL) while IL-10 secretion had an inverse profile. By the same token, Mϕ2 also produced 7 times more IL-10 (1,436 ± 396.6 pg/mL) than Mϕ1 (185.1 ± 54.07 pg/mL). In the presence of *M. leprae*, Mϕ2 cells increased the IL-10 production (2,302 ± 539 pg/mL) in relation to Mϕ1 cells (361.8 ± 156.5 pg/mL) (Fig. 1D-E).

Efferocytosis increases* M. leprae *uptake by Mϕ1 macrophages

Chronic evolution of infectious diseases is thought to be associated with macrophage reprogramming toward an Mϕ2 profile, particularly in those diseases associated with Th2 responses. Consequently, the capacity of *M. leprae* to be internalized by monocyte-derived macrophages was evaluated by both electron microscopy and flow cytometry. Our data showed that Mϕ2 internalized significantly more *M. leprae* when compared with Mϕ1 (Mϕ1: 0.84 ± 0.2% *vs.* Mϕ2: 3.78 ± 1.2%) (Fig. 2A-C).

The next step involved investigating whether phagocytosis of ApoJ or ApoN cells modulate *M. leprae* internalization. In this context, our results showed that there is an increase in the percentage of internalized *M. leprae* in the presence of apoptotic cells by Mϕ1 when compared with macrophages stimulated with *M. leprae* alone ($p<0.05$) or those maintained in the presence of viable cells stimulated with the mycobacteria ($p<0.05$) (Fig. 3A-C). The presence of apoptotic cells did not affect mycobacterial

uptake by Mϕ2 cells as evaluated by flow cytometry (Fig. 3A-C). There was no difference in the uptake of *M. leprae* in the presence of ApoJ or ApoN cells (Fig. 3C).

Phagocytosis of apoptotic cells in the presence of M. leprae shifts Mϕ1-polarized cells towards Mϕ2 phenotype

Since phagocytosis of apoptotic cells modulated *M. leprae* uptake in Mϕ1 cells, we evaluated whether this augmented phagocytic capacity was accompanied by phenotypic changes. We, therefore, evaluated the expression of CD163 and SRA-I by flow cytometry. Apoptotic cell uptake did not significantly affect CD163 and SRA-I expression in Mϕ1 cells. However, in the presence of *M. leprae*, apoptotic cells significantly increased the expression of both CD163 (5.20 ± 0.59 in ML+apo *vs.* 1.35 ± 0.46 in non-stimulated cells) and SRA-I (26.53 ± 6.84 in ML+apo *vs.* 2.57 ± 0.41 in non-stimulated cells) (Fig. 4A-B, Fig. S2). Besides, an increase in *M. leprae* viability as measured by the 16S rRNA/16S rDNA ratio was detected in Mϕ1 cells (Fig. 4C). In Mϕ2 macrophages, the uptake of apoptotic cells did not change the CD163 and SRA-I patterns when compared with non-stimulated cells despite the presence of *M. leprae* (Fig. 4A-B, Fig. S2).

The supernatants of Mϕ1 macrophages contained significantly lower levels of IL-6 and IL-15 produced in response to *M. leprae* after apoptotic cell phagocytosis when compared to cells stimulated with *M. leprae* alone (Fig. 5A-B). The production of IL-10 rose after phagocytosis of apoptotic cells in the presence of *M. leprae* in contrast to *M. leprae*-stimulated cells. TGF-β secretion in the presence of *M. leprae* increased after phagocytosis of apoptotic cells compared to non-stimulated cells and those stimulated with *M. leprae* and live Jurkat. In Mϕ2 macrophages, neither *M. leprae* nor apoptotic cells affected the IL-6, IL-15 or IL-10 expression. *M. leprae* and apoptotic

cells in the presence or absence of *M. leprae, however*, were able to induce increased levels of TGF-β in relation to non-stimulated Mϕ2 cells (Fig. S3).

Arginase contributes to induction of the Mϕ2 phenotype in* M. leprae*-treated Mϕ1 cells in the presence of apoptotic cells

Previous reports have described arginine as the essential substrate driving macrophage polarization (35). Alterations in local L-arginine metabolism, principally mediated by the enzymes arginase (Arg) and inducible nitric oxide synthase (iNOS), have been linked to pathological wound healing. In order to investigate the activation of arginase during leprosy, we analyzed the skin lesions of patients with the polar forms of the disease We were able to demonstrate that, in multibacillary patient skin lesions, large numbers of macrophages express arginase. In contrast, in paucibacillary lesions, few cells express this enzyme (Fig. 6A). Over subsequent years, interest in Arg/iNOS has focused on the classical versus alternatively-activated (Mϕ1/Mϕ2) macrophage paradigm (36-37). We found an increase in arginase 1 mRNA expression in Mϕ1 cells stimulated with *M. leprae* and apoptotic cells in relation to both non-stimulated cells and those that had received these stimuli separately (Fig. 6B). We tested whether the arginase blockade could impair the Mϕ1-Mϕ2 phenotype shift by using nor-NOHA. It was seen that pre-treatment with nor-NOHA impaired a rise in the percentage of SRA-I-expressing cells in Mϕ1 (Fig. 6C). The expression of CD163 (data not shown) and IL-15 (Fig. 6D) was not affected by the arginase blockade although lower IL-10 levels in nor-NOHA pre-treated cells stimulated with apoptotic cells and *M. leprae* were observed (Fig.6E).

Effect of apoptotic cell phagocytosis and* M. leprae *stimulation on T cell priming by Mϕ1 cells

We then determined whether the phenotypic shift in Mϕ1 cells after apoptotic cell clearance in the presence of *M. leprae* could affect T cell priming. Mϕ1 cells were treated with *M. leprae* in the presence or absence of apoptotic cells for 24h. The cell cultures were then stimulated with autologous $CD3^+$ T cells (1 MΦ1: 10 T cells) for 48h (Fig. 7A). The cytokine profile in the culture supernatants was subsequently evaluated; and it was found that the Th2 cytokines IL-4 and IL-13 increased in response to *M. leprae* in cultures stimulated with apoptotic cells (Fig. 7B-C) but not the IFN-γ cytokine (Fig. 7D).

Discussion

Macrophages are the preferred targets for infection of intracellular pathogens, including mycobacteria. This microbe-host interaction can lead to the development of protective (microbicidal) or permissive (phagocytic) host-cellular programs (34), with the latter culminating in progression to active disease. In addition, macrophages can also undergo dramatic molecular and functional changes upon encounter, interaction with, and the uptake of apoptotic cells (38). The elucidation of mechanisms behind macrophage activation has recently provided important insights into various physiological and pathological conditions (39-40).

It was shown, for example, that macrophages differentiated *in vitro* in the presence of GM-CSF (Mϕ1) are pro-inflammatory and microbicidal, promoting cellular immunity. On the other hand, macrophages differentiated in the presence of M-CSF (Mϕ2) are unable to activate $CD4^+$ T cells, even after treatment with the CD40 ligand and IFN-γ (13, 41-43). Again, the present study investigated the role played by efferocytosis in the modulations of macrophage programs and, possibly, in the pathogenesis of leprosy.

Recent data from our group suggest that the skin lesion macrophages of multibacillary patients and Mϕ2 macrophages have a similar phenotype, with high expressions of CD163 and IDO (30, 44). This observation is reinforced here by the demonstration of intense arginase expression in lepromatous patient lesions. The phenotype of paucibacillary patient macrophages are equivalent to the classically-activated ones (Mϕ1) (30, 44) despite the fact that few positive cells for Mϕ2 markers are present in the skin lesions of these patients (30, 44), suggesting that the maintenance of lower numbers of Mϕ2 cells at the paucibacillary infection site may sustain infection in this group. Our data reinforce this hypothesis, showing that even though both Mϕ1

and Mϕ2 macrophages are able to internalize *M. leprae*, Mϕ2 macrophages boast a greater phagocytic capacity when compared to Mϕ1 cells.

Previous studies have demonstrated that Mϕ2 macrophages are able to bind to the surface apoptotic cells at 4°C and to phagocytose them at 37°C at a higher percentage than Mϕ1 macrophages (45). Moreover, Verreck and colleagues demonstrated that Mϕ2 can internalize more BCG than Mϕ1 (13). However, Makino and colleagues reported no difference in the internalization of BCG by these two types of macrophages (42). A recent work showed that macrophage differentiation in the presence of M-CSF showed a greater phagocytic capacity to internalize lymphoma target cells opsonized with rituximab in comparison to GM-CSF-induced cells. Furthermore, the addition of IL-10 significantly increased, while IL-4 greatly decreased phagocytosis in both M-CSF- and GM-CSF-differentiated macrophages (45). These findings reinforce the hypothesis that paucibacillary patients exhibit a predominance of Mϕ1-like macrophages and that, conversely, multibacillary patients exhibit a predominance of Mϕ2-like macrophages in their respective lesions.

Several studies have related the phagocytosis of apoptotic cells with the internalization of microorganisms. Apoptotic induction of lymphocytes by *Trypanosoma cruzi* and the phagocytosis of apoptotic cells by macrophages increase predisposition to the parasite, suggesting that the phagocytosis of apoptotic cells plays a role in disease persistence (46). Similarly, *Leishmania* sp. infection induces apoptosis in neutrophils, which are subsequently engulfed by macrophages. These apoptotic cells serve as a "Trojan Horse" so to speak in that the recognition of apoptotic neutrophils prevents contact of the parasite with the macrophage receptors. As a result, *Leishmania* is able to reach its final host, the macrophage, culminating in the establishment of infection (47,48). Our model found that Mϕ2 macrophages naturally phagocytose more

M. leprae when compared to Mϕ1. However, in the presence of apoptotic cells, there is an increase in *M. leprae* uptake by Mϕ1 but not by Mϕ2 cells. Jurkat cells and neutrophils were used as sources of apoptotic cells, at which time similar results were observed.

In Mϕ1 macrophages, apoptotic cells and *M. leprae* increased the expression of scavenger receptors CD163 and SRA-I, shown to be specific markers for Mϕ2 macrophages. This suggests that stimulation with *M. leprae* in the presence of apoptotic cells is altering the phenotypic profile of this population. Moreover, in the presence of *M. leprae*, the phagocytosis of apoptotic cells by Mϕ1 macrophages resulted in reduced secretion of the pro-inflammatory cytokines IL-6 and IL-15 and increased production of such anti-inflammatory molecules as IL-10, TGF-β, and arginase. Arginase has been described as a marker of alternative macrophage activation, exercising a crucial host-protective function by down-regulating excessive Th1-induced inflammation in different experimental models (49).

Previous studies have shown increased mRNA and protein IL-15 expression in macrophages of paucibacillary when compared to those of multibacillary patients (34). IL-15 may represent a key cytokine involved in granuloma formation and may enhance cellular immune responses against mycobacterial antigens (15, 50). Our data showed that macrophage differentiation *in vitro* with GM-CSF induced increased levels of IL-15, reinforcing our hypothesis that these cells exhibit the phenotype of macrophages found in paucibacillary patients. The reduction of IL-15 could drive the TGF-β increase, indicating that in paucibacillary patients the increased percentage of apoptotic cells contributes to a possible reversal of the macrophage phenotype, which allows the establishment of infection even in the presence of the cellular immune response. In the

presence of *M. leprae*, stimulation with apoptotic cells increased the levels of IL-10, implying a polarization of these macrophages towards the phagocytic pathway.

Arginase has emerged as a key player in the mammalian immune system; and it is known that this enzyme is involved in various aspects of inflammation. We have found that the blockade of arginase *in vitro* impairs increased SRA-I and IL-10 production in Mϕ1 cells stimulated with both *M. leprae* and apoptotic cells. Arginase induction is not specific to *M. leprae* stimulation. In fact, previous studies have demonstrated that both apoptotic cells and their derivatives may alter the physiology of macrophages towards a regulatory phenotype by reducing nitric oxide production (51, 52). In addition, others have demonstrated that mycobacteria-infected macrophages produce soluble factors, (i.e IL-10), which can induce arginase expression in an autocrine-paracrine manner (53). The data presented here suggest that arginase not only increases in the Mϕ2 population, but is also involved in Mϕ2 differentiation. To date, even though the molecular biology of arginase regulation in the various macrophage subsets has been poorly studied, a possible regulatory role for SOCS1 has been described (54).

Our lymphocytic stimulation assay clearly demonstrated that efferocytosis by Mϕ1 macrophages induced a Th2 response against *M. leprae* mediated by IL-4 and IL-13, two cytokines that may contribute to the alternative macrophage activation. It can be hypothesized that the increased TNF induced by early-stage *M. leprae* infection may be responsible for the higher frequency of apoptotic cells in skin lesions. Efferocytosis contributes to the maintenance of Mϕ2 cells in skin lesions, which, in turn, reinforces the maintenance of *M. leprae* in paucibacillary lesions. Interaction with these newer Mϕ2 cells with naïve T cells tends to intensify a Th2 response that might lead, in later stages, to Mϕ2 differentiation at the infection site. Altogether, these data suggest that *M.*

444 *leprae*-induced apoptosis and/or TNF contribute to the formation of a favorable
445 microenvironment for the establishment of infection in paucibacillary patients
446 notwithstanding the presence of an effective cellular immune response.

447

448 **Legends**

449 **Fig. 1 - *M. leprae* stimulation did not alter the phenotype of macrophages**
450 **differentiated *in vitro*.** To determine whether *M. leprae* may induce a different
451 phenotype in macrophages differentiated *in vitro*, $CD14^+$ cells from healthy donors were
452 stimulated with M-CSF (50 ng/mL) or GM-CSF (50 ng/mL) for 6 days to obtain Mϕ1
453 or Mϕ2 macrophages (A), respectively. Cells were stimulated with irradiated *M. leprae*
454 at 10 µg/mL for 24h after which the expression of CD163-APC (B) and SRA-I-PE (C)
455 were evaluated by flow cytometry. Concentrations of the pro-inflammatory cytokine IL-
456 15 (D) and the anti-inflammatory cytokine IL-10 (E) in the supernatants were evaluated
457 by ELISA. Experiments were performed at least twice in triplicate and data were
458 presented as mean ± SD. # $p< 0.05$ in relation to Mϕ1. * $p< 0.05$ in relation to
459 Mϕ1+ML. Bars = 50 µm.

460 **Fig. 2 - Mϕ2 cells differentiated *in vitro* are more phagocytic than Mϕ1.** (A)
461 Ultrastructural analyses were performed to evaluate whether there are differences in the
462 phagocytic capacity of these cells differentiated *in vitro*. Mϕ1 or Mϕ2 cells were
463 stimulated with irradiated *M. leprae* at 10 µg/mL for 2h and *M. leprae* uptake was
464 analyzed by electron microscopy (A and B) or flow cytometry (C). Experiments were
465 performed at least three times in triplicate and data were presented as mean ± SD. Red
466 arrows point to *M. leprae* in vacuoles inside Mϕ1 and Mϕ2 cells. # $p< 0.05$ in relation to
467 Mϕ1. Bars = 2 µm (A) or 1 µm (B).

468 **Fig. 3 - The presence of apoptotic cells increases *M. leprae* uptake by Mϕ1 cells.**
469 Ultrastructural analyses were performed to evaluate *M. leprae* uptake by Mϕ1 (A and B)
470 and Mϕ2 cells in the presence of apoptotic cells (irradiated Jurkat cells - ApoJ or

irradiated neutrophil - ApoN). Mϕ1 or Mϕ2 cells were stimulated with irradiated *M. leprae* at 10 µg/mL for 2h in the presence or absence of apoptotic and live Jurkat cells or neutrophils (1:1). The percentage of *M. leprae* uptake was analyzed by flow cytometry (C). Experiments were performed at least three times in triplicate and data were presented as mean ± SD. # $p< 0.05$ in relation to Mϕ1. * $p< 0.05$ in relation to ML, LiveJ+ML or LiveN+ML-stimulated Mϕ1 cells. A full arrow shows *M. leprae* inside cells whereas dashed arrows indicate an apoptotic Jurkat cell inside Mϕ1 macrophage. Bars = 2µm.

Fig. 4 - Phagocytosis of apoptotic cells in the presence of *M. leprae* shifts Mϕ1 polarization towards a Mϕ2 phenotype. To determine whether *M. leprae* stimulation in the presence of apoptotic cells could modulate the cell phenotype, Mϕ1 and Mϕ2 cells were stimulated with irradiated *M. leprae* at 10 µg/mL for 24h in the presence or absence of apoptotic Jurkat cells (1:1). CD163-APC (A) and SRA-I-PE (B) expression were evaluated by flow cytometry and the percentages of positive cells were shown. (C) *M. leprae* viability was determined by the ratio of 16S rRNA/ 16S rDNA in Mϕ1 cells stimulated or not with apoptotic cells following 24h of infection. Experiments were performed at least three times in triplicate and data were presented as mean ± SD. * $p< 0.05$ in relation to non-stimulated (N.S) Mϕ1 cells and Mϕ1+ApoJ. *** $p< 0.001$. #$p<0.05$ in relation to N.S and ML-stimulated Mϕ1 cells.

Fig. 5 - Apoptotic cell uptake changes the cytokine secretion pattern induced by *M. leprae* in Mϕ1 cells. Mϕ1 cells were stimulated with irradiated *M. leprae* at 10 µg/mL for 24h in the presence or absence of apoptotic or live Jurkat cells (1:1) and the concentrations of IL-6 (A), IL-15 (B), IL-10 (C), and TGF-β (D) in cell supernatants

were evaluated by ELISA. Experiments were performed at least three times in triplicate and data were presented as mean ± SD. *p < 0.05, ***p<0.001.

Fig. 6 - The increased SRA-I expression in Mϕ1 cells stimulated with apoptotic cells and *M. leprae* is dependent on arginase. Arginase expression in leprosy patient skin lesions (BT, n=5; LL, n=5) was evaluated by immunoperoxidase. The images are representative of one BT and one LL patient (A). Mϕ1 cells were stimulated with irradiated *M. leprae* at 10 µg/mL for 24h in the presence or absence of apoptotic or live Jurkat cells (1:1); and the arginase 1 expression was evaluated by real time PCR. # p< 0.05 in relation to N.S., beads, ML, LiveJ and LiveJ+ML groups. (B). Mϕ1 cells were stimulated with irradiated *M. leprae* at 10 µg/mL for 24h in the presence or absence of apoptotic Jurkat cells (1:1) or arginase inhibitor nor-NOHA at 10 µM. SRA-I-PE expression was evaluated by flow cytometry. #p<0.05 in relation to N.S., vehicle, ML and ApoJ groups. *p<0.05 (C); and the concentrations of IL-15 and IL-10 in the cell supernatants were evaluated by ELISA (D, E). Experiments were performed at least three times in triplicate and data were presented as mean ± SD. # p< 0.05 in relation to N.S., beads, ML, LiveJ and LiveJ+ML groups. * p< 0.05.

Fig. 7 - Efferocytosis leads Mϕ1 cells to induce Th2 responses to *M. leprae* antigens *in vitro*. Mϕ1 cells were stimulated with irradiated *M. leprae* at 10 µg/mL for 24h in the presence or absence of apoptotic Jurkat cells (1:1). Cells were incubated with $CD3^+$ T cells (TLϕ) (1 Mϕ1: 10 TLϕ cell) for 48h and cell supernatants were harvested for cytokine analysis (A). IFN-γ (B), IL-13 (C), and IL-4 (D) concentrations in cell supernatants were determined by ELISA. Experiments were performed at least three times in triplicate and data were presented as mean ± SD. *p < 0.05 in relation to *M. leprae*-stimulated cells.

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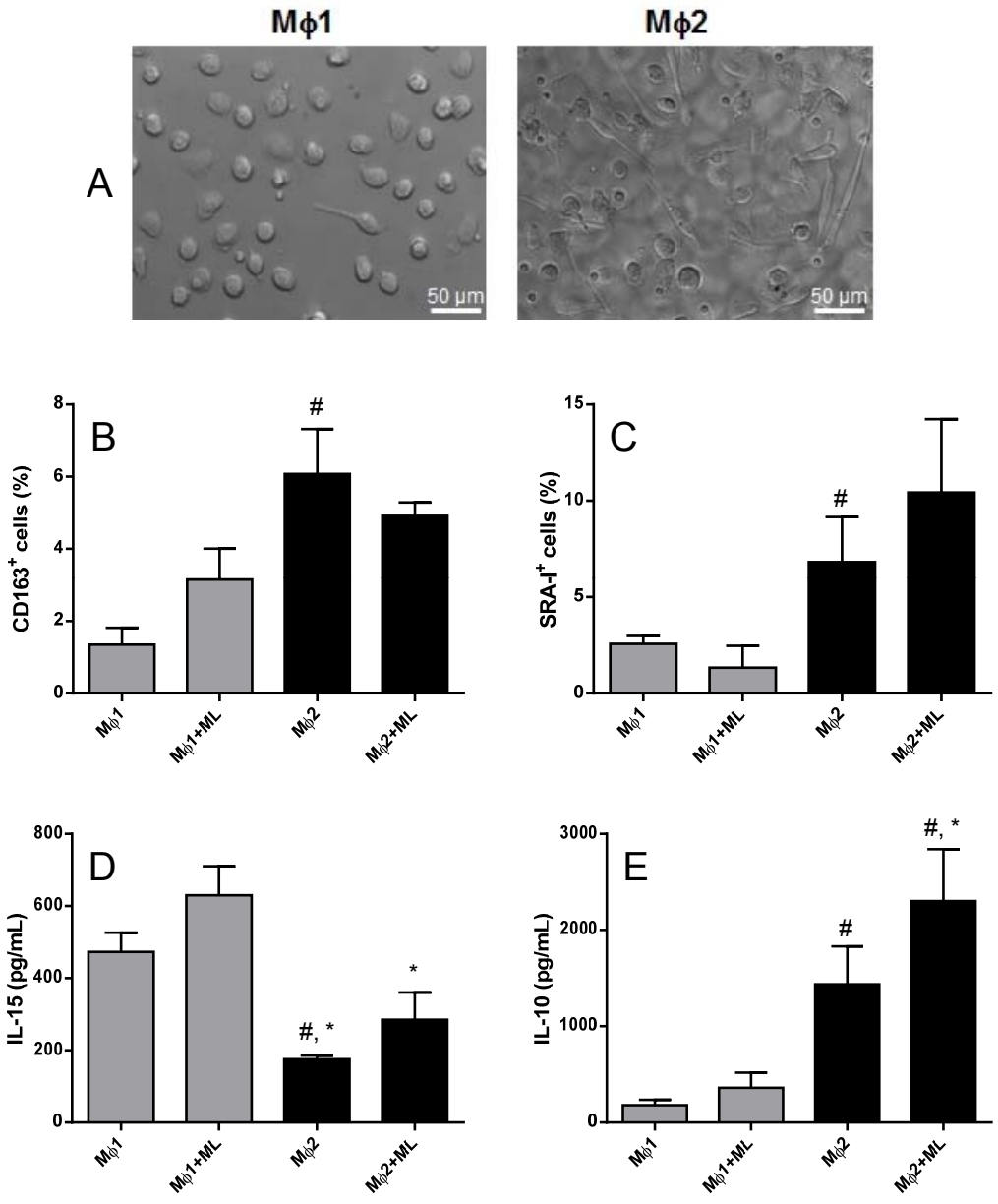
Figure 1



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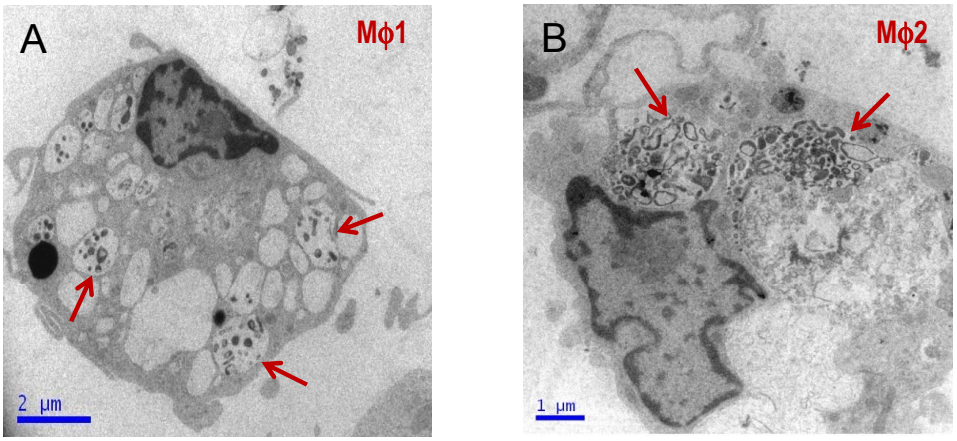

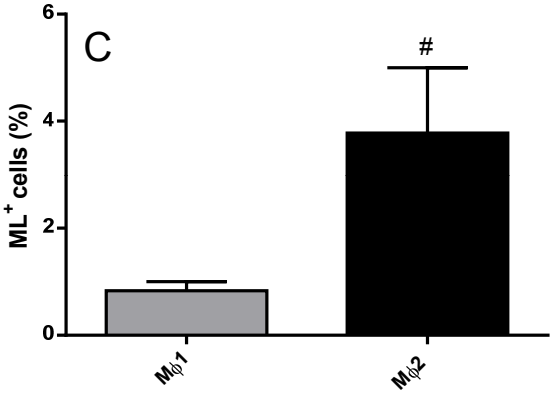

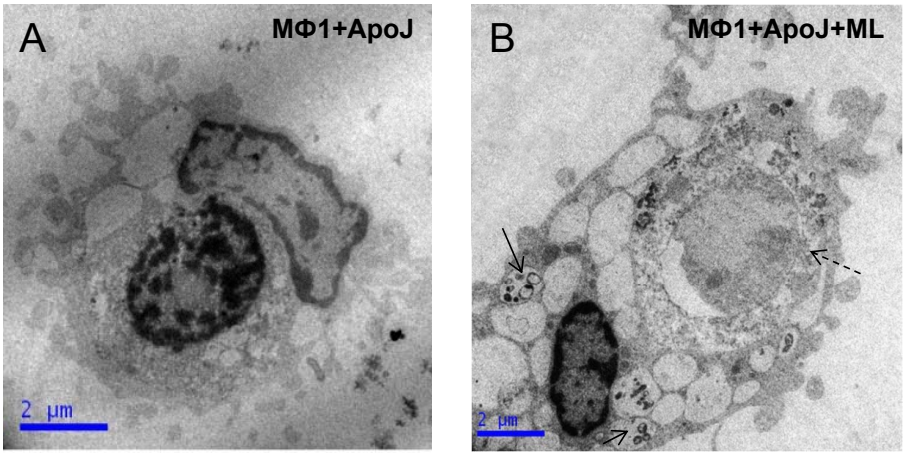

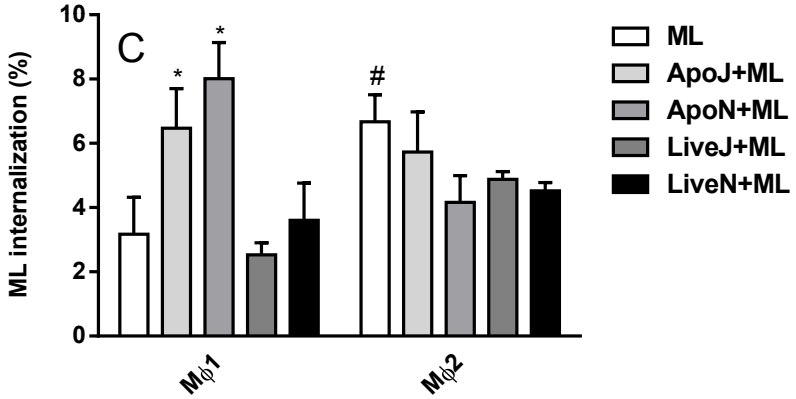

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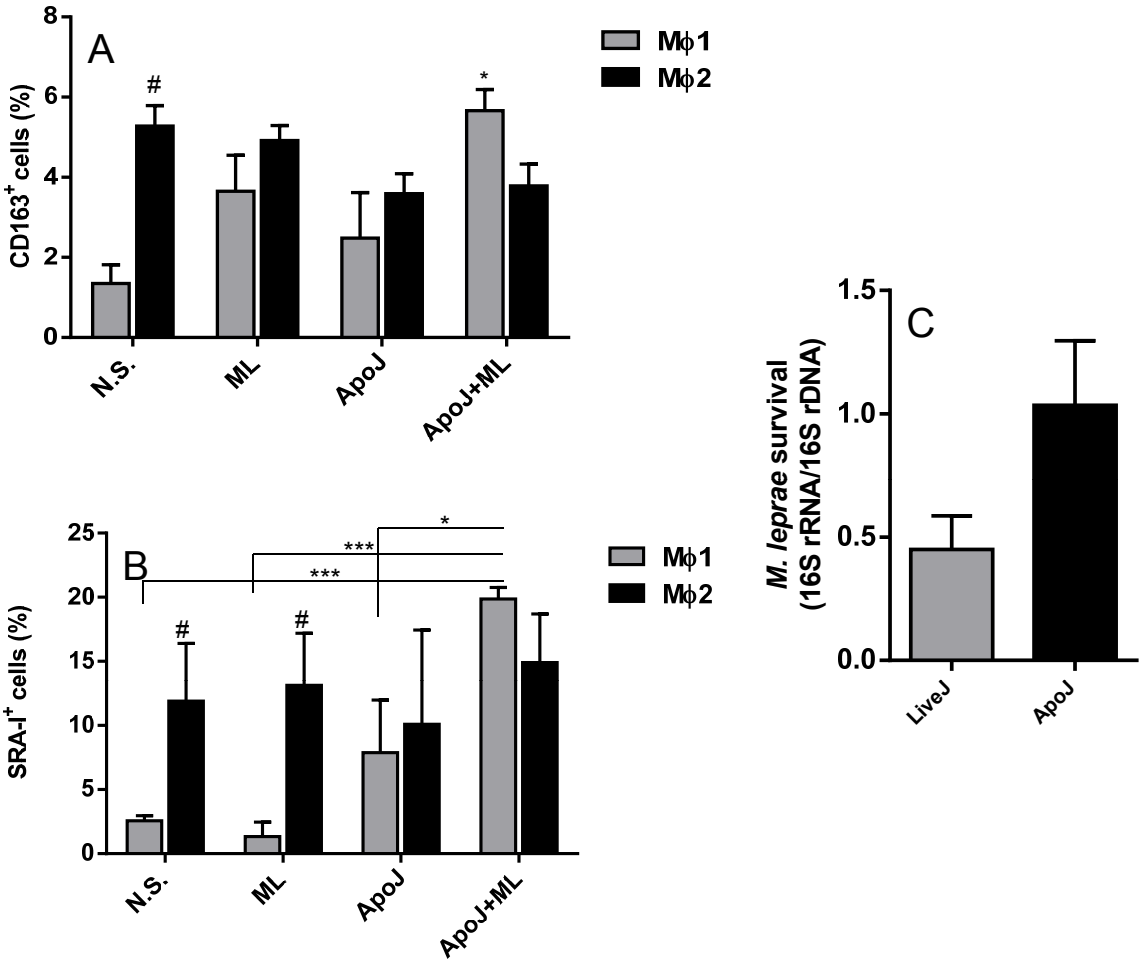

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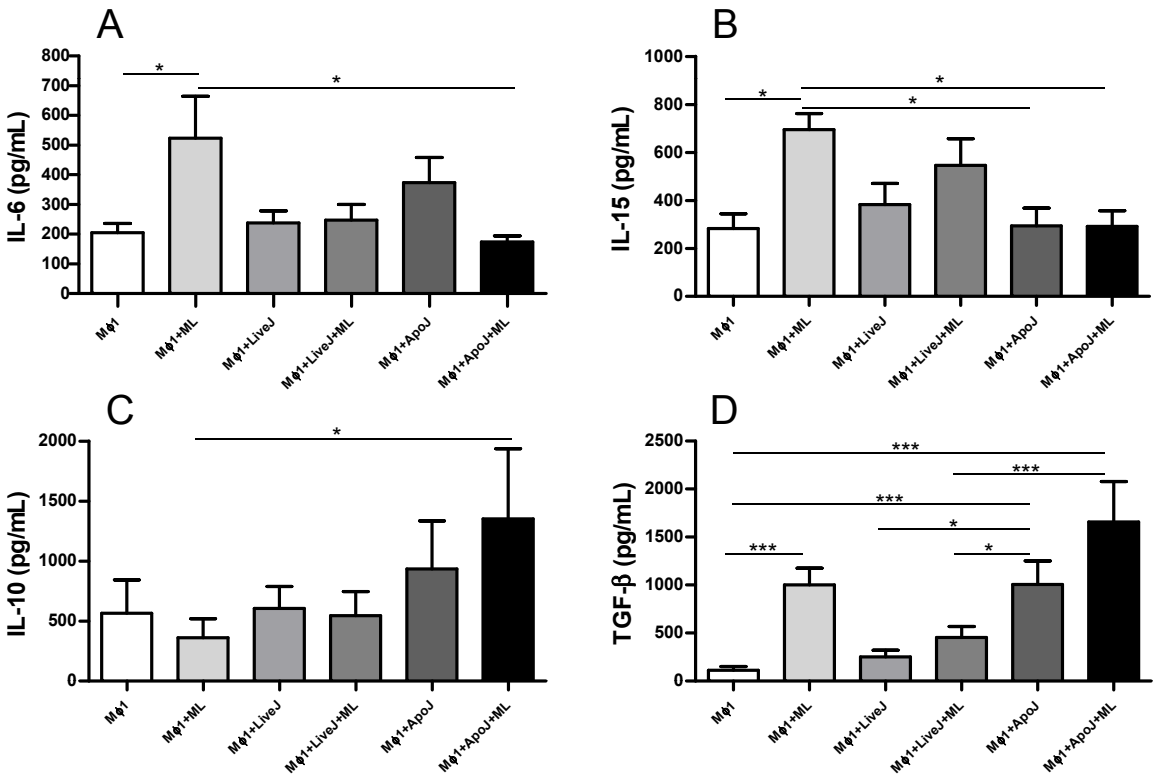

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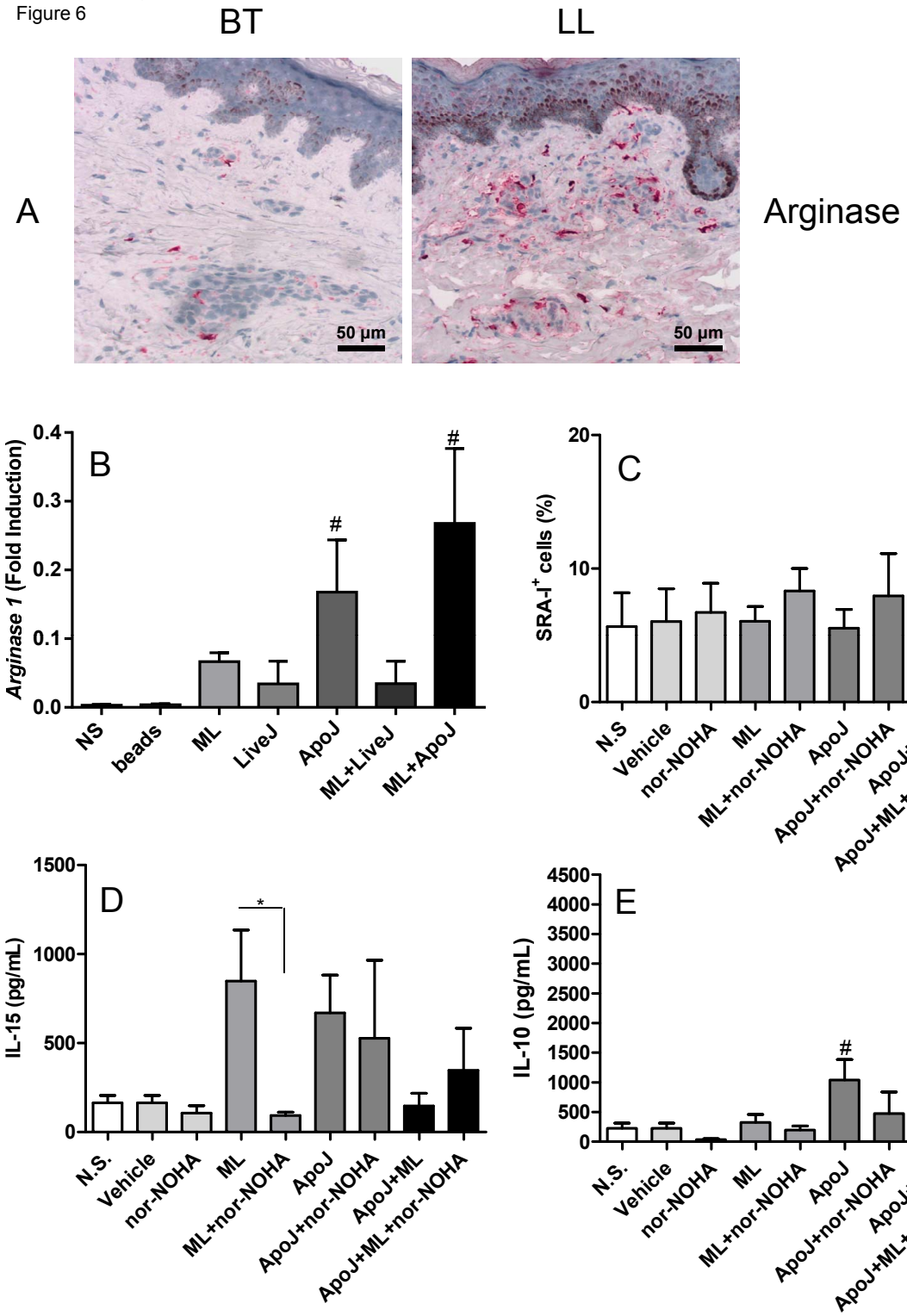

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Figure 7