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# The M1/M2 Pattern and the Oxidative Stress are Modulated by Low-Level Laser in Human Macrophage

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## Abstract

**Objective:** This study investigated the phototherapy (PhT) effect on U937 cells activated by lipopolysaccharide (LPS) from *E. coli*. PhT has been reported to control the release of inflammatory mediators from different cells activated by LPS. It is unknown if the macrophage M1/M2 pattern as well as the reactive oxygen species (ROS) generation and the pro- and anti-inflammatory cytokines secretion from U937 cells can be influenced by PhT. Methods: The U937 cells, a human monocytic cell line, were cultured and matured to macrophages in a medium with LPS and irradiated (660 nm) at 4.5 J/cm$^2$. Apoptosis was standardized with Annexin-V and propidie iodate (PI) and the cytotoxicity assay evaluated by MTT. ROS was measured by DCFH-DA. The cytokines, chemokines, NF-κB and Sp1 activity were measured by ELISA. PhT was studied in the presence of a Sp1 inhibitor, mithramycin. Results: The pro-inflammatory cytokines and chemokines, ROS and NF-κB were downregulated by PhT. On the contrary, IL-10, arginase, PGC-1β and glutathione were upregulated. The Sp1 activity was increased after PhT to values higher than those from cells only LPS-treated; oppositely the mithramycin abrogated this effect. Conclusion: The PhT restored the macrophage polarization toward M2 pattern as well as balanced the oxidative stress and modulated the immune response upregulating the IL-10 secretion by a mechanism in which Sp1 transcription factor has a crucial role.

**Keywords:** U937 cells; Oxidative stress; M1/M2 pattern; Cytokines; Chemokines; Sp1; NF-κB; Phototherapy

## Introduction

One of the main functions performed by activated macrophages is the ingestion of foreign agents and their destruction by the production of reactive oxygen species (ROS) [1]. During the phagocytic process, macrophages play essential roles in inflammation and immune responses. Inflammatory cytokines and nitric oxide are known to be involved in various inflammatory diseases such as inflammatory bowel disease, rheumatoid arthritis and sepsis [1-3].

Polarized macrophages by Th1 cytokines are called M1 macrophages and are considered as classically activated. M1 macrophages are pro-inflammatory and one of its main features is to be dependent on LPS and IFNγ to be activated. In addition it is able to secrete pro-inflammatory mediators such as TNF, IL-1β, IL-6 and nitric oxide [4,5]. The polarized macrophages by Th2 cytokines are known as M2 macrophages and are activated by an alternative pathway on influence of IL-4, IL-13, IL-10 and glucocorticoids [6]. M2 macrophages present increased manose and galactose receptors expression, ornithine and polyamines secretion from arginase pathway, besides of secreting a diverse repertoire of chemokines [6].

Lipopolysaccharide (LPS), a component of the membrane of Gram-negative bacteria, is one of the most potent activators of macrophages [7]. LPS activates gene transcription by binding to its membrane receptor, TRL4, on circulating monocytes, inducing signal transduction pathways leading to the phosphorylation of kinases. These in turn activate various transcription factors, including the AP-1 families and the NF-κB [8,9].

It has been shown that an oxidized cytosolic environment amplifies the activation of NF-κB, which plays a critical role during inflammatory processes by activating many genes encoding for pro-inflammatory cytokines and immunoregulatory mediators [10]. It has also been suggested that ROS influence mechanisms that are dependent on NF-κB [11]. In the course of many inflammatory diseases reduced levels of glutathione (GSH) and/or increased levels of free radicals have been detected indicating the involvement of oxidative stress [12,13].

Some reports highlight the activation of stimulatory protein-1 (Sp1) transcription factor as the responsible by IL-10 generation in physiological and pathological conditions [14-16]. Some authors have shown that the rising of oxidative metabolism in epithelial cells increases Sp1 binding which allows to induce the IL-10 in turn inhibits the transcription of mediators responsible for initiating and perpetuating the inflammatory environment in the cell [17,18].

Phototherapy (PhT) has been used in the treatment of pleurisy [19], asthma [20,21] and lung emphysema [22,23]. Although it is well established that macrophages are important cells in the development of acute lung inflammation, the *in vitro* PhT effect on these cells after LPS stimulation has not yet been elucidated.

Therefore, the goal of the present study was to investigate the phototherapy effect on macrophage polarization and oxidative stress in human macrophage activated by *E. coli* lipopolysaccharide.

## Material and Methods

### Cells and culture conditions

The human monocytic cell line U937 (American Type Culture Collection, Rockville, MD, USA) was cultured in RPMI 1640 medium (Sigma-Aldrich Co. St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FCS), 100 U/mL penicillin and 100 $mg.mL^{-1}$ streptomycin at 37°C with 5% $CO_2$ in a humidified incubator. U937 cell line was incubated at 37°C with 5 nm of 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma Co.). Once seventy-two hours of incubation with TPA had elapsed, the U937 monocytic cell line was differentiated to macrophage-like cells as outlined described by [24]. The expression of different PDE isoenzymes of these macrophage-like phenotype cells closely resembles those human lung macrophages from bronchoalveolar lavage (BAL) [25,26]. Cells were centrifuged and resuspended in fresh media in 24-well plates at a concentration of $500 \times 10^3$ cells/per well, 24 hr before experimental use.

### Cell treatment and experimental design

U937 cells were plated in serum-free RPMI-1640 prior to the addition of LPS (500 $ng.mL^{-1}$) and then were incubated with lipopolysaccharide from *E. coli* during 12 hours. For antioxidant treatment, cells were incubated with 5 $ng.mL^{-1}$ of N-acetylcysteine (NAC) 1 hr before the LPS stimulation. In order to investigate whether the phototherapy effect on cytokines release involves a cellular signaling through NF-κB, the effect of low-level laser on NF-κB protein was tested and measured in U937 cells by the enzyme linked immunoassay (ELISA) method, 12 hr after LPS exposure. In order to evaluate the involvement of Sp1 transcription factor in IL-10 produced in U937 cells treated with LPS, we used mithramycin (100 nM) as a Sp1 specific inhibitor 1 hr before the LPS stimulation.

### Phototherapy

A diode laser with an output power of 30 mW and operating in 660 nm (model: MM Optics) irradiated a total area of 0.785 $cm^2$ for 60 seconds, which resulted in an energy dose of 4.5 $J/cm^2$. The optical power was calibrated utilizing a Newport Multifunction Optical Meter model 1835C. The stability of the laser output during the experiment was monitored by collecting light with a partial reflecting surface (4%). For irradiation, the cover plate was removed and the U937 cells from each well were irradiated once, 1 hr after the addition of LPS

### MTT-based cytotoxicity assay

The assay with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is dependent upon a reduction of the tetrazolium salt by mitochondrial dehydrogenase in viable cells, which forms a blue Formosan product (Mosmann 1983). Briefly, 20 µL of MTT solution was added to each well, and the plates were kept in an incubator in 5% $CO_2$ at 37°C for 12 hours and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and 100 µL of dimethyl sulfoxide was added per well. After keeping this mixture in the dark at room temperature for 30 minutes, absorbance was measured at a wavelength of 570 nm.

### Measurement of Apoptosis

Twelve hours after exposure to LPS, human macrophage were washed with PBS and stained with Hoechst 33258 and propidium iodide (PI) according to manufacturer's protocol. Briefly, human macrophages were incubated with 10 $\mu g.mL^{-1}$ PI for 10 minutes at 37°C in the dark, and then incubated with 5 $\mu g.mL^{-1}$ Hoechst 33258 for 5 minutes. The cells were washed twice with PBS and then examined under a fluorescence microscope using a filter with an excitation wavelength of 330-385 nm and an emission wavelength of 420-480 nm. Apoptotic cells were stained with blue fluorescence, while necrotic cells were stained with red fluorescence.

### Intracellular reactive oxygen species (ROS) generation

To investigate whether the phototherapy modulates the LPS-induced intracellular ROS generation in U937 cells, the 2',7'-dichlorofluorescin diacetate (DCFH-DA) (MoBiTec, Göttingen, Germany) was used for ROS detection. DCFH-DA diffuses into the cell and is hydrolyzed by intracellular esterases to polar 2',7'-dichlorofluorescin. This non-fluorescent fluorescin can be oxidized to the highly fluorescent 2',7'-dichlorofluorescein by intracellular oxidants. U937 cells were cultured to adhere on the plates and incubated with 10 µM DCFH-DA for 30 min. Afterwards the cultures were washed twice with RPMI 1640 and, subsequently treated as previously described. Fluorescence baseline was measured with a fluorimeter (FLUOstar, BMG LabTechnologies, Offenburg, Germany) immediately after wood dusts were added, as it was described by Imrich and Kobzik [27]. After 12 h of incubation at 37°C and in 5% $CO_2$ and 21% $O_2$, the fluorescence was measured again. The results are given as percentage change from baseline values.

### Measurement of nitric oxide

Nitric oxide (NO) production was measured as nitrite (a stable metabolite of NO) concentrations using the Griess reagent system. The fluorogenic substrate of 2,3-diaminophthalenen (DAN) can be changed into the fluorescent 1-(H)-naphotiazolei with $NO_2$- under the acid solution. U937 cells were washed twice with cold PBS, then suspended in 1 mL PBS, and incubated with 10 µL of DAN at a concentration of 0.5 $mg.mL^{-1}$ at 37°C in the dark for 40 additional minutes. After the incubation, 50 µL of 2.8 NaOH were added to finish the reaction. Considering that a portion of NO can be converted to nitrate mainly in the presence of oxidative stress, we have also measured the nitrate concentration. The fluorescent intensity of the cell suspensions was then monitored by a fluorescence spectrophotometer with an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

### Measurement of intracellular gluthatione (GSH)

Reduced GSH was assayed using the method described by Hissin and Hilf [28]. U937 cells were inoculated into six-well plates; the number of cells was $2 \times 10^5$ cells/well. After the incubation, living and dead cells were examined by trypan blue staining and the cell viability was calculated. The medium was discarded and 0.4 mL of 5% trichloroacetic acid (TCA) was added. After 30 minutes of incubation at 4°C to extract GSH, 50 mL of TCA extract were added to tubes containing 1 $mg.mL^{-1}$ o- phthalaldehyde (1 mL) in 50 $mmol.L^{-1}$ phosphate/5 $mmol.L^{-1}$ EDTA buffer (pH 8.0). The tubes were incubated at 37°C in the dark for 15 minutes. Fluorescence was measured by a fluorescence spectrophotometer in which the excitation

and the emission wavelengths were 485 nm and 550 nm, respectively. The concentration of GSH was determined from a GSH standard.

### Measurement of cytokines and chemokines

Pro- and anti-inflammatory mediator levels in U937 cells were determined by the Enzyme Linked Immuno Sorbent Assay (ELISA) using commercially available kits according to the manufacturer's instructions. TNF-α was determined by a human-specific sandwich immunoassay kit obtained from R&D Systems (Minneapolis, MN, USA). Both the interleukins (-1β, -6, -8 and -10) and chemokines (CCL2 and CXCL10) concentrations were determined using a sandwich ELISA kit, which used peroxidase and tetramethylbenzidine as a detection method (BioRad, USA). The detection limit of those assays was found to be in the range of 1-5 $pg.mL^{-1}$. The protein data in U937 cells supernatant were expressed as $pg.mL^{-1}$.

### Real time-PCR

Elapsed twelve hours of LPS, U937 cells were removed and processed to RT-PCR. For this, total RNA was isolated from cells by TRIzol reagent (Gibco BRL, Gaithersburg, MD), according to the manufacturer´s protocol. RNA was subjected to DNase I digestion, followed by reverse transcription to cDNA. PCR was performed in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems, Foster City, CA) using the SYBRGreen core reaction kit (Applied Biosystems). Primers used for TNF mRNA quantification were forward 5'- AAATGGGCTCCCTCTATCAGTTC-3' and reverse 5'-TCTGCTTGGTGGTTTGCTACGAC- 3'; for HIF-1α mRNA measurement the primers used were forward 5'-TGGCTCCCTATACCCAATGG-3' and reverse 5'-TGGCAGTGGCAGTGATGGTAG-3'; the primers for IL-1β were forward 5'- CACCTCTCAAGCAGAGCACAG -3' and reverse 5'-GGGTTCCATGGTGAAGTCAAC -3'; for IL-6 mRNA quantification, the primers were forward 5'- TCCTACCCCAACTTCCAATGCTC-3' and reverse 5'- TTGGA TGGTCTTGGTCCTTAGCC-3'; the primers for IL-8 were forward 5′ TTAGCACTCCTTGGCAAAACTG -3′ and reverse 5'- CTGGCCGTGGCTCTCTTG 3', and the primers for IL-10 mRNA quantification were forward 5'-TGACAATAACTGCACCCACTT - 3' and reverse 5' TCATTCATGGCCTTGTAGACA; the primers for arginase mRNA quantification were forward 5'-GGAAGAGTCAGTGTGGTGCTGG-3' and reverse 5'-CAGGGAGAAAGGACACAGGTTGC-3'; the primers for PGC-1β, arginase were forward 5'-GGCAGGTTCAACCCCGA-3' and reverse 5'-TTGCTAACATCACAGAGGATATCTTG-3'; and for β-actin the primers 5'- AAGTCCCTCACCCTCCCAAAAG-3' and reverse 5'-AAGCAATGCTCACCTTCCC-3' were also used. Quantitative values for TNF, IL-1β, IL-6, IL-8, IL-10, HIF-1 arginase, PGC-1β, and β-actin mRNA transcription were obtained from the threshold cycle number, where an increase in the signal associated to an exponential growth of PCR products began to be detected. Melting curves were generated at the end of every run to ensure product uniformity. The relative target gene expression level was normalized on the basis of β-actin expression as endogenous RNA control. ΔCt values of the samples were determined by subtracting the average Ct value of TNF, IL-1β, IL-6, IL-8, IL-10, HIF-1 arginase, and PGC-1β mRNA from the average Ct value of the internal control β-actin. As it is uncommon to use ΔCt as a relative data due to this logarithmic characteristic, the $2^{-\Delta Ct}$ parameter was used to express the relative expression data [29]. Results are expressed as a relative ratio to the sum of β-actin transcript level as internal control.

### Measurement of NF-κB protein and p65 subunit phosphorylation

For measuring NF-κB protein, U937 cells were plated and allowed to adhere for 2 hr. After washing off the non-adherent cells, cultures were incubated in 0.25% FCS RPMI 1640 and treated for 12 hr with LPS (10 $\mu g.mL^{-1}$). Cultures were washed again to remove the LPS and incubated in serum free RPMI 1640 in the absence or presence of NAC or laser for 1 hr. Culture supernatants were aspirated and the cells were lysed by incubation for 20 min with 0.1 M HCl (22°C), followed by a disruption using a cell scraper. Posteriorly, the intracellular content of NF-κB protein was determined using NF-κB ELISA kit by R&D System following the manufacturer's recommendations. Phosphorylated NF-κB p65 (RelA) concentration was determined by using a colorimetric cell-based ELISA (Active Motif; Carlsbad, CA, USA).

### Measurement of Sp1 activity

The assessment of Sp1 activity was determined in nuclear extracts from U937 cells with the TransAM ELISA Kit (Active Motif, CA, USA) according to the manufacturer´s instructions. Briefly, U937 cells lysates were centrifuged (15,000 × g, 30 min, 4°C), and the supernatant (nuclear extract) was collected for an evaluation of the Sp1 activity. Absorbance was determined at 450 nm using a microplate reader (Bio-Tek Instruments, Synergy™ H4, Winooski, VT).

## Statistical Analysis

Comparisons were performed using two-tailed paired t test or Mann-Wittney U test when appropriate. All the analyses were performed using a statistic software package (GraphPad Prism) and the results were considered significant when $p<0.05$.

## Results

### Cytotoxicity

In order to determine the influence of pharmacological inhibitors, N-acetylcysteine (NAC) or mythramicyn, and the incubation period on viability of human macrophages, the cells were incubated with these agents and the results evidenced that both the NAC and the mythramicyn did not interfere significantly on cellular viability in the concentrations studied in the present manuscript. We did not show the effect of PhT on human macrophages, but likewise the pharmacological inhibitors the PhT did not alter the cellular viability. The data are represented in Table 1.

| Inhibitors concentration | Cellular variability | |
|---|---|---|
| NAC | 20 mM | 93.7 ± 12 |
| BMS205820 | 4 μM | 96.8 ± 20.1 |
| Mithramycin | 100 nM | 89.4 ± 15 |

Table 1: MTT-based cytotoxicity assay of inhibitors from the following pathways in U937 cells: ROS, NF-κB, and Sp1. U937 cells were incubated for 1 hour with N-acetylcysteine (20 mM) or mythramicyn (100 nM) or medium, and the cellular viability was assessed by using the MTT reduction assay, as described in the "Methods" section.

Results are mean ± SD of one representative experiment in triplicates. Statistical differences were considered when $p<0.05$.

## Apoptosis

Some energy densities delivered to cell or tissue can exacerbate the mitochondrial activity with consequent exaggerated production of ROS which would induce cell to initiate the apoptosis process. In other cases, a misguide dose of laser can induce a cellular membrane disruption with loss of cytoplasmic content. Thereby, the assessment of both the apoptosis and necrosis index aimed to investigate if the dose of PhT chosen in the present study could interfere on cell death by itself or in the presence of stimuli, such as LPS used herein. The Figure 1 shows a marked increase of apoptosis percentage induced by staurosporine (8 μM), a positive control for annexin V binding. Similarly to staurosporine, the U937 cells that were incubated with LPS presented a significant increase of apoptosis with more cells binding to annexin than U937cells that were not incubated with LPS. The Figure 1B illustrates the U937 cells that were incubated with LPS presented a non-significant increase of necrosis with few cells binding to PI when compared to human macrophage did not expose to LPS. Otherwise, the PhT on U937 cells that were exposure to LPS did not modify significantly the necrosis percentage in supernatant. Likewise, the PhT did not induce necrosis in human macrophage in comparison to those cells that were not exposed to laser.

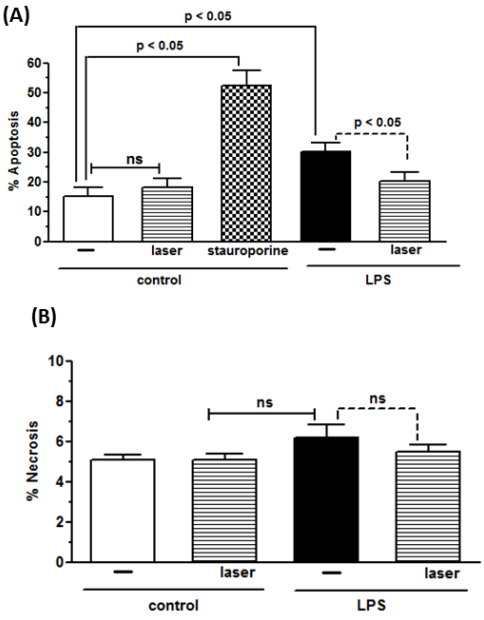


**Figure 1:** Effect of phototherapy on apoptosis index. Percentage of U937 cells binding annexin V after twelve hours of LPS (1 μg.mL$^{-1}$) exposure was used as the apoptosis index. U937 cells were treated with staurosporine (8 μM) as a positive control for annexin V binding. One hour after LPS addition in the culture medium, the U937 cells were irradiated with phototherapy (4.5 J/cm$^2$) during 60 seconds. Three hours after LPS addition, ROS level was measured. Results are expressed as fluorescence units. Each bar represents mean ± S.D. of three independent experiments. Statistical differences were considered when $p<0.05$.

## Macrophage pattern

The cells of the monocyte-macrophage lineage are characterized by diversity as well as by plasticity. In response to various stimuli, macrophages can be subjected to classical activation or alternative, which depends on the Th1/Th2 response from T cells. Therefore, we examined whether PhT was able to exert effect on macrophage M1/M2 polarization induced by LPS. As illustrated in the Figure 2, the U937 cells exposure to LPS presented an increase of (M1 pattern) pro-inflammatory chemokines secretion CCL2, CXCL10 and HIF-1α when compared to U937 cells from control group (2A). On the contrary, the (M2 pattern) anti-inflammatory mediators secretion IL-10 (6I and 6J), arginase and PGC-1β was markedly decreased in LPS-activated U937 cells (2B); however the phototherapy restored the pro- and anti-inflammatory balance shifting the macrophage immune response toward a M2 pattern as well as decreasing the pro-inflammatory chemokines secretion classically recognized as M1 pattern.

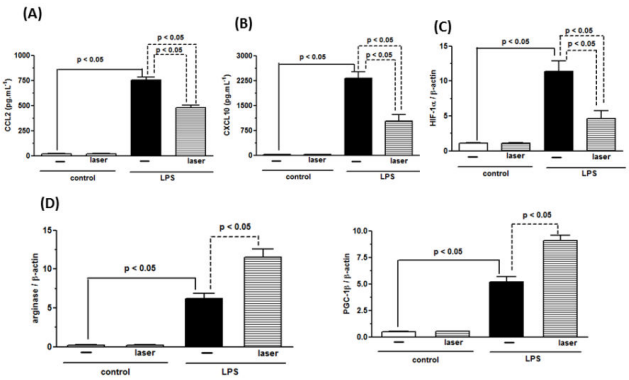


**Figure 2:** Effect of phototherapy on M1/M2 pattern. U937 cells were stimulated with LPS (1 μg.mL$^{-1}$) and treated with phototherapy (4.5 J/cm$^2$) during 60 seconds. One hour before the stimulation with LPS, U937 cells were pretreated with 20 mM N-acetylcysteine (NAC). Twelve hours after LPS addition, the M1 mediators (CCL2, CXCL10 and HIF-1α) and M2 mediators (arginase-1 and PGC-1β) levels in U937 cells were measured. Results are expressed as fluorescence units. Each bar represents mean ± S.D. of three independent experiments. Statistical differences were considered when $p<0.05$.

## Intracellular ROS

There is a close relationship between the cell activation induced by LPS and increased secretion of ROS. The cellular activation induced by LPS can unbalance the oxidative stress metabolism stimulating cells to secrete oxidant agents. Besides, it is well known that great amount of ROS induces apoptosis. For that reason and considering that PhT reduced the apoptosis percentage in LPS-stimulated U937 cells, we assessed if the PhT attenuates the ROS secretion. We used NAC as positive control for inhibiting the ROS secretion. The Figure 3 shows an increase of the intracellular ROS level in LPS-stimulated U937 cells. The ROS intracellular concentration has significantly dropped in cells that were treated with LPS and irradiated with PhT when compared to U937 cells only activated by LPS. The NAC antioxidant agent was as efficient as the phototherapy. It was observed that phototherapy or NAC has not had any effects on ROS level in U937 cells not bathed with LPS.

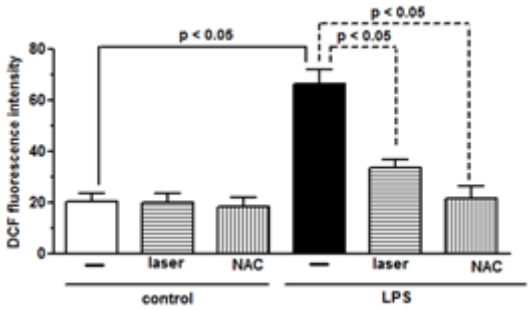


**Figure 3:** Effect of phototherapy on ROS level. U937 cells were stimulated with LPS (1 μg.mL$^{-1}$) and treated with phototherapy (4.5 J/cm$^2$) during 60 seconds. One hour before the stimulation with LPS, U937 cells were pretreated with 20 mM N-acetylcysteine (NAC). Twelve hours after LPS addition, the ROS level in U937 cells was measured. Results are expressed as fluorescence units. Each bar represents mean ± S.D. of three independent experiments. Statistical differences were considered when p<0.05.

## Nitric oxide (NO)

The inflammatory response to LPS in macrophages includes initial induction of ROS and induction of inducible nitric oxide synthase (iNOS). This last step would lead to the production of NO in toxic amounts. Some authors have evidenced increases in the NOS activity and the generation of NO in diverse cellular types and that it contributes to the worsening of the inflammatory process. In this sense, the PhT effect was evaluated on NO metabolites (nitrite and nitrate) in LPS-stimulated U937 cells. As shown in the Figure 4, the nitrite concentration in U937 cells supernatant was significantly increased in the LPS only-treated group. The Figure 4A illustrates that phototherapy significantly suppressed the increase of NO formation in comparison to the LPS-activated but not irradiated. The rise of NO concentration in U937 cells activated by LPS was markedly dropped by NAC more efficaciously than when U937 cells were treated with phototherapy. Considering that a portion of NO can be converted to nitrate, specially, in the presence of oxidative stress, we also measured the nitrate concentration (4B). The phototherapy effect on the nitrate production was very similar to the one observed in the nitrite production. It was found that there is no modification in the NO (nitrite or nitrate) production in supernatant cells, when the phototherapy or NAC is applied on U937 cells that were not stimulated with LPS.

## Glutathione (GSH)

The exposure of inflammatory cells to LPS stimulates the exaggerated ROS secretion through activation pathway via of NF-κB. This oxidant environmental repressed the genes responsible for the transcription of anti-oxidant elements. Hence emerge an unbalance of pro- and anti-oxidant agents. Moreover, in the course of many inflammatory diseases reduced levels of glutathione (GSH) and/or increased levels of free radicals have been detected indicating the involvement of oxidative stress. For that reason, the effect of laser on the redox status of human macrophage induced by LPS was determined by GSH level.

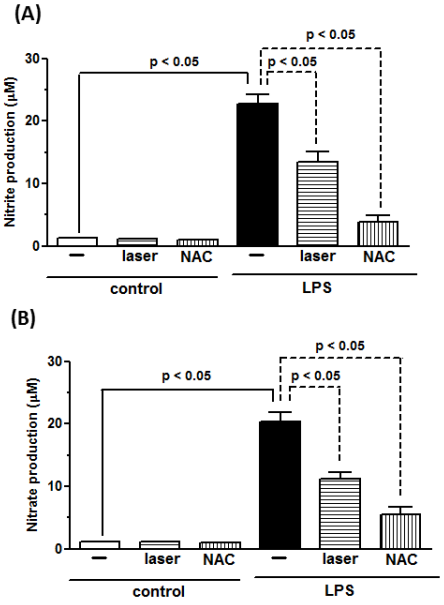


**Figure 4:** Effect of phototherapy on NO level. One hour after the addition of LPS (1 μg.mL$^{-1}$) in the culture medium, the U937 cells were irradiated with a fluency of 4.5 J/cm$^2$ during 60 seconds. U937 cells were pretreated one hour before the LPS with 20 mM N-acetylcysteine (NAC). Twelvw hours after LPS, the concentrations of nitrite (4A) or nitrate (4B) were measured using the Griess reagent system. Each bar represents mean ± S.D. of three independent experiments. Statistical differences were considered when p<0.05.

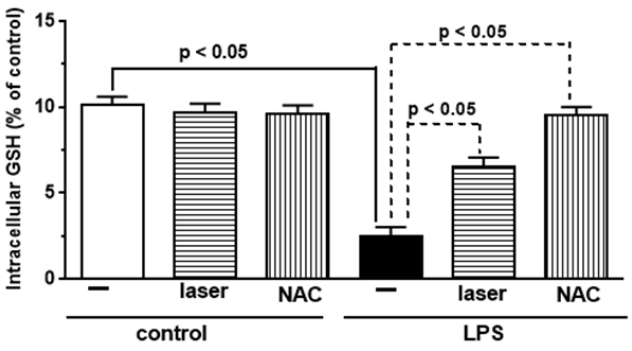


**Figure 5:** Effect of phototherapy on GSH level. U937 cells were activated with LPS (1 μg.mL$^{-1}$) and one hour after its addition in the culture medium the cells were treated with phototherapy (4.5 J/cm$^2$) during 60 seconds. In another assay, one hour before the LPS addition, U937 cells were pretreated with 20 mM N-acetylcysteine (NAC) and twelve hours after it, they were cultured in the presence of LPS. The GSH concentration was determined by fluorescence from a GSH standard. Each bar represents mean ± S.D. of three independent experiments. Statistical differences were considered when p<0.05.

As illustrated in Figure 5, the GSH level dramatically decreased when the cells were stimulated with LPS compared to U937 cells that were not activated by LPS. When pretreated with NAC or treated with phototherapy, GSH depletion was greatly decreased in the LPS-activated U937 cells compared to LPS-stimulated U937 cells but not treated with NAC or phototherapy. Phototherapy or NAC in U937 cells that were not stimulated by LPS has not had any effects on GSH level.

## Cytokines

Just like the oxidative unbalance, the equilibrium between the pro- and anti-inflammatory responses of cytokines secreted by U937 cells is disturbed in the presence of LPS. LPS activates gene transcription by binding TRL4 on circulating monocytes, inducing signal transduction pathways leading to the phosphorylation of kinases that activate various transcription factors responsible for pro-inflammatory cytokines synthesis, as for example TNF, IL-1β, and IL-8. Besides there is a marked decline of genes activation that control transcription factors producers of anti-inflammatory cytokines, such as IL-10. The IL-10 presents elevated levels in the plasma and in the tissue of animal models of acute inflammation and it inhibits the release of pro-inflammatory cytokines (TNF, IL-1β, IL-6 and IL-8) from monocytes/macrophages, thus preventing subsequent tissue damage. In order to evaluate the ability of PhT in modulating the balance between the pro- and anti-inflammatory proteins secretion, the LPS-treated U937 cells supernatant was assessed as well as the mRNA expression for each cytokine (Figure 6).

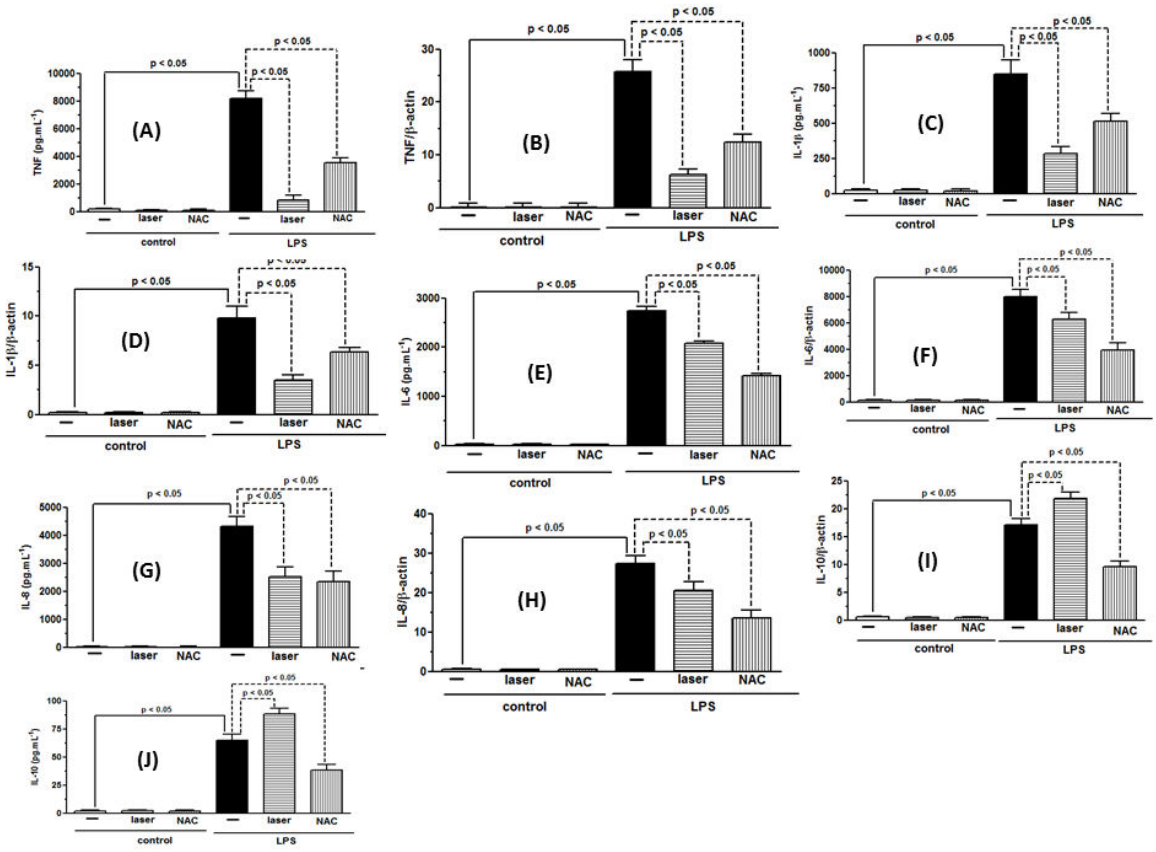


**Figure 6:** Effect of phototherapy on cytokines. Supernatant from U937 cells cultured with LPS (1 μg.mL$^{-1}$) and treated or not with phototherapy (4.5 J/cm$^2$) were collected in order to analyze the mRNA and the protein concentration of TNF (A and B), IL-1β (C and D), IL-6 (E and F), IL-8 (G and H) (pro-inflammatory interleukins) and IL-10 (I and J) (anti-inflammatory interleukin). The cells received laser therapy one hour after the addition of LPS in the culture medium. One hour before LPS, U937 cells were pretreated with 20 mM N-acetylcysteine (NAC). Twelve hours after U937 cells stimulation with LPS the mRNA and the protein concentration of inflammatory mediators were determined by Enzyme Linked Immuno Sorbent Assay (ELISA) and RT-PCR, respectively. Each bar represents mean ± S.D. of three independent experiments. Statistical differences were considered when $p<0.05$.

No effects of NAC and phototherapy were detected on the expression of any of the cytokines studied in U937 cells not challenged with LPS. This basal value was considered the background for further analyses. For TNF (6A), IL-1β (6C), IL-6 (6E), IL-8 (6G), and IL-10 (6I) the mRNA or AM supernatant proteins TNF (6B), IL-1β (6D), IL-6 (6F), IL-8 (6H), and IL-10 (6J) were upregulated after the exposition to LPS when compared to U937 cells that were not bathed with LPS. Concerning the TNF and IL-1β, LPS-activated U937 cells showed markedly lesser mRNA expression and protein level of both cytokines after phototherapy in comparison with those cells that were LPS-stimulated but were not treated with NAC or phototherapy. Regarding the phototherapy effect on IL-8 and IL-6 secretion in U937

cells, the results indicate that although being significantly efficient, the laser therapy has not markedly reduced the expression and the cell supernatant protein level for both cytokines. For anti-inflammatory IL-10, the laser effect was the opposite of what was observed for pro-inflammatory cytokines, because the phototherapy has increased the mRNA expression and the protein concentration of this cytokine to values above those reached with LPS.

## IL-10 in U937 cells pretreated with NAC

Although the ROS concentration modulates the IL-10 secretion from inflammatory cells in different experimental models, it has never been reported whether the laser can increase the secretion of IL-10 from U937 cells by itself or exposed to LPS in a ROS-independent manner. Hence, we investigated the PhT effect on IL-10 level in supernatant from LPS-activated U937 cells is dependent on ROS generation. U937 cells were pretreated with N-acetylcysteine (NAC) to guarantee that the oxidative stress does not interfere with the phototherapy effect on IL-10. As can observed in Figure 7, there is a reduction of IL-10 level in supernatant of LPS-stimulated cells pretreated with NAC when compared with LPS-activated cells but not pretreated with NAC (as shown in Figure 6I). Nevertheless, the IL-10 protein level in supernatant from LPS-activated cells was higher than the control. Even in the presence of NAC, the level of IL-10 in supernatant from LPS-stimulated U937 cells treated with laser was kept high, similarly to the U937 cells not treated with NAC. The presence of NAC did not alter the IL-10 concentration in U937 cells from groups that were not stimulated with LPS.

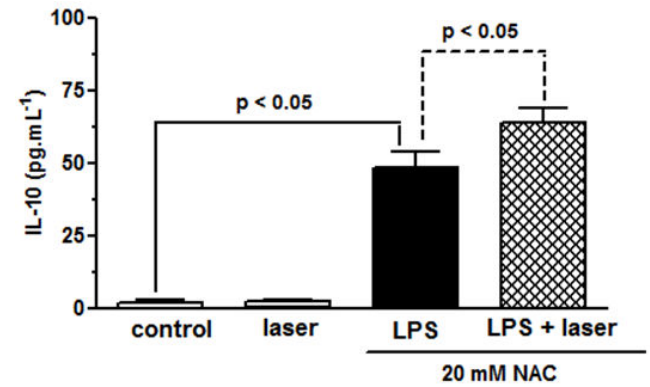


**Figure 7:** Effect of phototherapy on anti-inflammatory cytokine IL-10 in presence on N-acetylcysteine. Supernatant from U937 cells cultured with LPS (1 μg.mL$^{-1}$) and treated or not with phototherapy (4.5 J/cm$^2$) were collected in order to analyze the protein concentration of IL-10. Before the exposure to LPS or LPS plus laser, the U937 cells were treated with 20 mM N-acetylcysteine (NAC). Twelve hours after the U937 cells stimulation with LPS, the IL-10 protein concentration was determined by Enzyme Linked Immuno Sorbent Assay (ELISA) using kit. Each bar represents mean ± S.D. of three independent experiments. Statistical differences were considered when p<0.05.

## Sp1 transcription factor

Our results evidence that the laser effect on IL-10 secretion from human macrophages is not modulated by ROS, in this sense some reports highlight that it involves the activation of the stimulatory protein-1 (Sp1) transcription factor that is responsible for the IL-10 generation in physiological and pathological conditions. Some authors have shown that the rise of oxidative metabolism products in type II alveolar epithelial cells increases Sp1 binding, as well as downstream the transcription, which means that the Sp1 activation induces the IL-10 production which in turn inhibits the transcription of mediators responsible for initiating and perpetuating the inflammatory environment in the cell. Thus, we decided to investigate whether the PhT effect in LPS-stimulated U937 cells can involve the activation of the transcription factor that regulates the IL-10 anti-inflammatory protein expression. For this purpose, the Sp1 activity was measured. Figure 8 shows that the LPS markedly increased the Sp1 activity in comparison with those observed for both the control and the laser groups. When the U937 cells exposed to LPS were treated with phototherapy, the Sp1 was increased to values higher than those obtained in the presence of LPS. Oppositely, the pretreatment with NAC reduced the Sp1 activity in LPS-activated U937 cells.

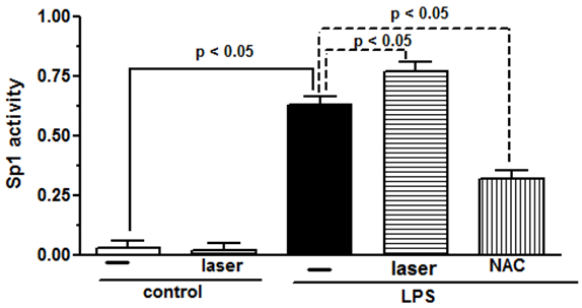


**Figure 8:** Effect of phototherapy on transcription factor Sp1 activity. The assessment of Sp1 activity was determined in supernatant (nuclear extracts) from U937 cells with the TransAM ELISA Kit. U937 cells were cultured with LPS (1 μg.mL$^{-1}$) and treated or not with phototherapy (4.5 J/cm$^2$) and then, the nuclear extracts were collected for evaluation of Sp1 activity. In another assay, one hour before the LPS, U937 cells were pretreated with 20 mM N-acetylcysteine (NAC). Twelve hours after the U937 cells stimulation with LPS the absorbance was determined at 450 nm using a microplate reader. Each bar represents mean ± S.D. of three independent experiments. Statistical differences were considered when p<0.05.

## IL-10 in U937 cells pretreated with mithramycin.

With the aim of confirming that the effect of phototherapy on the concentration of IL-10 in LPS-activated U937 cells is dependent upon the transcription factor Sp1, the U937 cells were pretreated with Sp1 specific inhibitor 1 hour before the LPS addition. Figure 9 represents a profound reduction of Sp1 activity in LPS-stimulated U937 cells pretreated with mithramycin. In addition, the pretreatment of LPS-activated U937 cells with mythramicyn abolished the phototherapy effect on IL-10.

## NF-κB protein and phosphorylated NF-κB p65

It is well established that the relation between the LPS and the toll-like receptor-4 can lead to the activation of NF-kB.

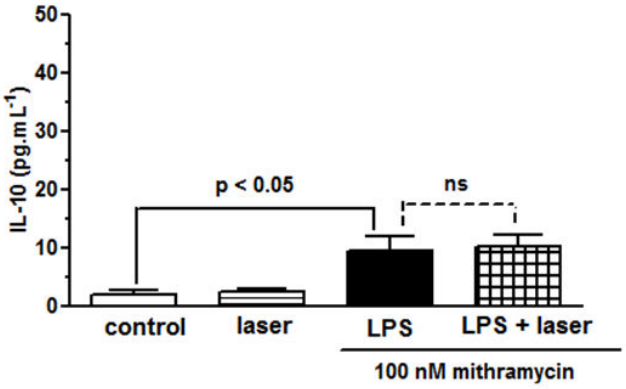


**Figure 9:** Effect of phototherapy on anti-inflammatory cytokine IL-10 in presence of mithramycin. U937 cells were preincubated with mithramycin (100 nM), a Sp1 specific inhibitor, and were activated with LPS (1 μg.mL$^{-1}$) or LPS plus phototherapy (4.5 J/cm$^2$). Twelve hours after the LPS exposure, the U937 cells supernatant was assayed by ELISA to determine the production of IL-10. Each bar represents mean ± S.D. of three independent experiments. Statistical differences were considered when p<0.05.

This transcription factor is responsible for much of the pro-inflammatory cytokines secreted by LPS-stimulated human macrophage. For that reason the PhT effect on NF-κB from U937 cells exposed to LPS was investigated. Figure 10A indicates that the protein concentration of nuclear transcription factor-κB was significantly increased by the challenge with LPS in comparison with cells from the control group. In Figure 10B is illustrated that LPS significantly increased the phosphorylation of the major trans-activating NF-κB domain p65 (RelA). On the other hand, the treatment with NAC or phototherapy markedly reduced the LPS-stimulated NF-κB domain p65 phosphorylation when compared to U937 cells that were not irradiated and not NAC treated. Phototherapy or NAC has not had an effect on both NF-κB domain p65 phosphorylation and NF-κB protein level in U937 cells that were not stimulated by LPS.

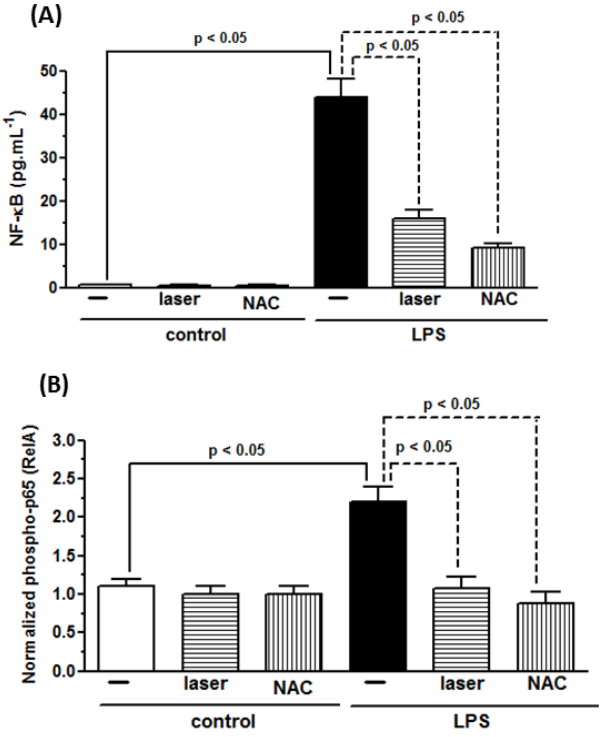


**Figure 10:** Effect of phototherapy on LPS-induced NF-κB activation. U937 cells cultured with LPS (1 μg.mL$^{-1}$) during three hours and treated or not with phototherapy (4.5 J/cm$^2$) were lysed and disrupted one hour after the LPS addition. U937 cells were incubated with NAC (20 mM) one hour before the addition of LPS in the culture medium. Twelve hours after LPS addition, the intracellular content of NF-κB protein (10A) was determined using NF-κB ELISA kit and the phosphorylated NF-κB p65 (RelA) (10B) concentration was determined using a calorimetric cell-based ELISA. Each bar represents mean ± S.D. of three independent experiments. Statistical differences were considered when p<0.05.

## Discussion

The present manuscript shows for the first time that the phototherapy (PhT) can modulate the secretion of pro- and anti-inflammatory cytokines that are features of macrophages M1/M2 polarization. Our results evidence that laser downregulates the cytokines secretion from type 1 polarized macrophages (pro-inflammatory), and oppositely upregulates the cytokines secretion from type 2 macrophages (anti-inflammatory). The oxidative stress was also modulated by PhT. Likewise that macrophage polarization, the laser also restores the balance between ROS and GSH in U973 cells, once it downregulated the ROS secretion and inversely upregulated the anti-oxidant activity of GSH. Moreover, we demonstrated that the effect of PhT in increasing the IL-10 anti-inflammatory cytokine secretion and it seems to involve the Sp1 transcription factor.

Curiously, the PhT was initially implemented in clinical practice by health professionals before the description of the laser-tissue interaction or action mechasnism.

The reason of using an *in vitro* experimental model is try to understand how the laser acts in human macrophages, i.e. the cellular signaling that allow to PhT modulates some processes since the transcription factor activation until secretion of inflammatory mediators. It is well known that the photons from laser are absorbed by mitochondria and it stimulates the generation of ATP [30, 31], however this action mechanism is not enough to explain the beneficial effect of PhT in both the experimental studies and clinical trials. Our results did not indicate whether the PhT has preference for a specific step of immune-inflammatory cascade in human macrophages activated by LPS. However, with the findings of this present study the comprehension of PhT effect on human macrophages can help to describe what better dosimetry is able to interfere efficiently in chronic disease. In fact, most of the studies focused on the laser effect on activated macrophages are seeking justifications that can explain further the beneficial effect of PhT in patients that are suffering from allergic asthma and chronic obstructive pulmonary disease.

The efficiency of PhT in attenuating the inflammation symptoms depends on light-cell/tissue interaction. It implies in considering that

the physical parameters of laser and the characteristics from each cell/tissue exposed to different conditions are crucial for a better treatment. In this sense, the PhT dosimetry should be adjusted to guarantee that the photobiomodulation happen. The electromagnetic spectrum has a "therapeutic window" that varies from 630 nm to 980 nm. Among this electromagnetic spectrum is possible to program the other laser parameters in accordance with cell/tissue type and thus it obtains a better laser light absorption. Based on physical parameters of laser light, it is important to stand out that it is possible to reach a beneficial effect *in vivo* using the same wavelength and energy density for treatment of a cell. It is due to the fact that the irradiation time and the mode are also fundamental to define the treatment. Therefore, the question is not about a stronger laser for *in vivo* treatment, but a setting for which maximum laser light can be absorbed. With respect to existence of a specific organ/body part targeted treatment or a whole body treatment in lung inflammation studies, the reports show that the beneficial effect of PhT on lung inflammation can occur with transcutaneous applications towards the main bronchus and in the axillary lymph nodes. Otherwise, when the *in vitro* experiments are performed the cells or tissue receive the irradiation directly on itself.

Among the groups interested in investigating the PhT effect on the stimulated macrophages stand out the studies that describe the PhT effect on cellular aspects such as the modulation of the mitochondrial activity of macrophages exposed to LPS [32]. These authors evidenced that laser with wavelength of 660 nm and energy density of 7.5 J/cm$^2$ or 780 nm with 3 J/cm$^2$ modulates the cellular activation status of macrophages in muscle inflammation. Other important topic of study that evidences the influence of laser on pro-inflammatory mediators using a laser diode with 780 nm and 3 J/cm$^2$ was shown by Souza and co-workers [33]. These authors showed that PhT attenuates the secretion of TNF but has no influence on MMP secretion. Some authors have investigated the PhT effect on aneurysm progression assessing the secretion of pro-inflammatory cytokines and the nitric oxide production [34]. These authors demonstrated that PhT with 780 nm and 2.2 J/cm$^2$ attenuate the cytokine secretion but upregulates the nitric oxide synthesis from LPS-stimulated macrophages. Our group has demonstrated a synergistic action of PhT (660 nm, 30 mW and 4.5 J/cm$^2$) with drug antioxidant NAC on MIP-2 mRNA expression from LPS- or $H_2O_2$-stimulated alveolar macrophages, and that the reduction of ROS intracellular generation as well as the NF-κB signaling seems to be involved [35]. We showed that PhT (660 nm, 30 mW and 4.5 J/cm$^2$) attenuates the cAMP dysfunction and the upregulation of TNF mRNA expression alveolar macrophages from bronchoalveolar lavage of mice exposed to LPS [36]. In an *in vitro* experimental model of resistance to treatment with corticoid where alveolar macrophages were exposed to LPS and $H_2O_2$, the PhT (660 nm, 30 mW and 4.5 J/cm$^2$) decreased IL-8 levels and increased the histone deacetylase (HDAC) activity as well as the response to steroid [37]. This result is surprising because the PhT can downregulates the IL-8 secretion, at the same time that it increases the HDAC activity through the activation of the protein kinase A via the inhibition of PI3K. This effect on HDAC does not allow that the steroid receptor becomes hyper-acetylated, and hence it restores the response to corticoid in reducing the IL-8 secretion from macrophages stimulated with LPS and $H_2O_2$. It maybe explains the fact of PhT reduced the IL-8 concentration even when the steroid receptor becomes unresponsive. We have demonstrated that PhT associated with anti-oxidant compound reduces the macrophage inflammatory protein-2 (MIP-2), mRNA expression and the generation of intracellular ROS in alveolar macrophages activated by LPS [38].

Based on the studies described above, we can affirm that PhT interacts with stimulated macrophages by LPS. Besides, these results indicate that the laser can influence different cellular signaling. Therefore, in the present manuscript the laser anti-inflammatory effect on LPS-stimulated U937 cells is also mediated, at least in part, by others chemical mediators, such as TNF, cAMP and MIP-2.

In the present manuscript we demonstrated that PhT restores both the equilibrium of oxidative stress metabolism and the polarization of macrophages toward M2 pattern. It is important to stand out that the laser doses were similar in almost all assays described herein. The dosimetry used in the present study is also in accordance to reports described. It's good news because it shows that the dosimetry of the laser can be characterized and correlated with its beneficial effect. The laser light is generated from photons, and when these photons are absorbed by biological tissues or by cells, more specifically the photoaceptors in mitochondria, these photons are converted into chemical energy. Therefore, there is no possibility to detect the laser light in biological fluids. This hinders the characterization of dose-response.

Concerning the apoptosis index, our results are in agreement with some authors who have evidenced a rise of apoptosis in different cellular types after the exposure to LPS [39-41]. In the present manuscript we demonstrated that phototherapy decreases the apoptosis index of U937 cells stimulated with LPS, which indicates that the phototherapy increased the time-life of a resident cell that has a pivotal role in initiating an acute lung inflammation, since that secretion of the pro-inflammatory cytokines, such as IL-8/KC, is fundamental to the neutrophil recruitment in the lung tissue. The phototherapy effect on apoptosis index can also be related to its effect in modulating the oxidative stress where the phototherapy reduced the ROS generation and increased the levels of glutathione. This equilibrium attenuates the inflammatory environment into U937 cells producing a more controlled immune response.

Macrophages are polarized into the pro-inflammatory direction by bacterial products or cytokines such as lipopolysaccharide (LPS), IFN-gamma and TNF, whereas they polarized towards an anti-inflammatory direction after exposure to IL-4, IL-10, IL-13, immune complexes or M-CSF [42]. Notably, the polarization pathways are highly dynamic and flexible, consisting of multiple intermediate states and subtypes [43]. There are few studies that focus on polarization activated macrophage. Some authors have demonstrated LPS enhanced M1-related cytokine and chemokine expression via mitochondrial biogenesis and histone modification, which may be a potent immune enhancing agent for the treatment of allergic diseases [44]. As described by Eichin and co-workers [45], our results also showed that LPS cause a disturbance in balance between the secretions of pro- and anti-inflammatory mediators from LPS-activated macrophages. The results presented herein also corroborate with these authors due to the fact that the LPS increases the secretion of cytokines and chemokines that are features of polarized macrophages toward M1 pattern, and oppositely it decreased the concentration of anti-inflammatory mediators that are markers of macrophage polarization toward M2 response. On the contrary, the PhT downregulated the pro-inflammatory cytokines secretion and inversely, it upregulates the anti-inflammatory cytokines secretion in U937 cells expose to LPS. These results evidence that PhT can modulate the M1/M2-related cytokines pattern into U937 cells activated by LPS in order to create a less inflammatory microenvironment.

It is well known that great amounts of ROS and the excessive activation of NF-κB nuclear transcription factor are features of inflammatory cells activated by different stimuli, including LPS [46]. Our results evidence that phototherapy produces a marked inhibitory effect on ROS generation and NF-kB activation.

The inflammatory response to LPS in macrophages includes initial induction of ROS and induction of inducible nitric oxide synthase (iNOS) and inflammatory cytokines. This last step would lead to the production of not only NO in toxic amounts, but also other cytokines [47,48]. Our results in the present study indicate that the PhT interferes in enzymatic machinery for synthesizing NO, since it decreased both the nitrite and the nitrate production in U937 cells exposed to LPS.

It has been reported that LPS, TNF-induced inflammation, or NF-κB activation are upregulated by the administration of agents that promote depletion of cellular GSH stores [49,50]. Our results corroborated with these authors since the GSH level in U937 cells was significantly dropped after the addition of LPS. In the current study we showed that PhT leads to a restoration of GSH levels in U937 cells challenged by LPS similarly to NAC. There is an interesting point in these results due to the fact that the PhT presented dual effects on the oxidative stress when U937 cells were activated by LPS, i.e., the phototherapy was able to decrease the ROS and NO levels at the same time that it increased the GSH levels, and possibly helped to reestablish the redox equilibrium of these cells.

The cytokine network related to the oxidation and the inflammation has attracted much interest and it is susceptible to modulation by anti-inflammatory compounds [51,52]. Our results showed that the cytokines TNF, IL-1β and IL-6 showed a similar increase in the mRNA expression as well as in the cellular supernatant protein after the activation of U937 cells by LPS and that the PhT reduced the pro-inflammatory cytokines in these cells. Concerning the participation of IL-6 and IL-8 in oxidative stress induced by LPS, our results showed that the PhT effect on IL-8 mRNA expression and protein level in U937 cells was similar to NAC. This is in agreement with the data provided by Ryan and co-workers [53], where they showed that LPS-stimulated IL-8 secretion can be blocked by NAC at the mRNA level as well as in the protein level.

Because ROS have been previously viewed as general messengers for signal-induced NF-κB activation [54], in this current study our results also showed the relevant suppressive effect of phototherapy on NF-κB. Our results coincide with findings from [55] where the phototherapy at a wavelength of 635 nm reduced both the ROS generation and the activation of NF-kB in LPS-stimulated human fibroblasts. In fact, we showed in the present manuscript, that the phototherapy effect on pro-inflammatory cytokines secretion and ROS generation can be the result of NF-kB inhibition. In addition, our results evidenced that laser does not work on Th2 cytokines and ROS from LPS-stimulated AM in the presence of NF-κB inhibitor. It means that the activation of transcriptional factor NF-κB is a target of anti-inflammatory effect of phototherapy.

Differently from pro-inflammatory cytokines studies herein, the IL-10 is an anti-inflammatory cytokine [56,57]. As expected, our results showed that the exposure to LPS increased the IL-10 level produced by U937 cells. Despite IL-10 is important for anti-inflammatory response, the PhT effect on the releasing of IL-10 in activated U937 cells has not been investigated. Surprisingly, our findings showed that the PhT induced an increase in IL-10 secretion in LPS-stimulated U937 cells stimulated. It suggests that the laser effect on IL-10 levels in LPS-exposed U937 cells can be dissociated of its effect on pro-inflammatory cytokines. This response can be an attempt of the phototherapy in counterbalancing the deleterious action of pro-inflammatory cytokines.

There is a close relationship between the products of oxidative stress metabolism and the expression of IL-10 [58]. Our results demonstrated that PhT keeps an increased level of IL-10 in LPS-activated U937 cells even in the absence of ROS. It was pharmacologically evidenced herein, when the U937 cells were pretreated with anti-oxidant drug, N-acetylcysteine, stimulated with LPS and posteriorly treated with laser. Some authors have shown that the rise of oxidative metabolism products in type II alveolar epithelial cells increases Sp1 binding, as well as downstream the transcription, which means that the Sp1 activation induces the IL-10 production which in turn inhibits the transcription of mediators responsible for initiating and perpetuating the inflammatory environment in the cell [59, 60]. The present manuscript evidenced by the first time that PhT can interfere with the transcription factor Sp1 since that the Sp1 activity in LPS-activated U937 cells was markedly increased by PhT indicating a Sp1-dependent laser effect on IL-10 secretion. This hypothesis was proved because the pretreatment of LPS-stimulated U937 cells with mithramycin, a Sp1 specific inhibitor, abolished the rise of IL-10 secretion induced by phototherapy. It means that in the absence of Sp1 transcription factor the laser does not upregulate the expression of IL-10 in a situation of oxidative stress. Thereby, these results lead us to believe that the phototherapy effect driven to increase the IL-10 secretion is via upregulation of Sp1 activity in these conditions in which the oxidative equilibrium was disturbed.

Finally, we conclude that PhT shifts the cytokines secretion from M1 type macrophages towards to M2 type macrophages. The PhT also restores the equilibrium of oxidative stress reducing ROS and inversely increasing GSH and attenuates pro-inflammatory cytokines secretion from LPS-activated human macrophages probably acting on phosphorylated NF-κB. Moreover, the PhT modulates the response driven to anti-inflammatory cytokines leading to an increased secretion of IL-10 by an action mechanism that depends on the Sp1 transcription factor.

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