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The Nitrone Spin Trap 5,5-Dimethyl-1-pyrroline N-oxide prevents M1-like Phenotypic Switch of Lipopolysaccharide-Primed Macrophages

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Abstract M1-like inflammatory phenotype of macrophages plays a critical role in tissue damage in chronic inflammatory diseases. M1-like macrophages produce reactive oxygen species, inflammatory cytokines (IL-1b, IFN-b), express inflammatory protein such as nitric oxide synthase (iNOS) and surface markers such as CD80; CD86; CD14; CD44. Because M1-like activation contributes to inflammation, decoding its mechanism may lead to find novel therapies. The nitrone spin trap DMPO reacts with free radicals to form adducts, thus reducing its chain reactions. Our studies have shown that DMPO has also anti-inflammatory effects that may not be related to its free radical trapping properties. Herein, we hypothesize that DMPO can reduce LPS-induced M1-like activation of macrophages by changing its transcriptome and proteome. To test this hypothesis we incubated RAW 264.7 cells with 1 ng/ml LPS in the presence or absence of 50 mM DMPO for 6h or 24h. Cells were used for the mRNA detection of M1-phenotypic molecular markers. Transcriptomic analyses are consistent with DMPO preventing the inflammatory M1-like of macrophages by reducing surface markers, inflammatory molecules and type-1 interferon signaling. To corroborate these data we used western-blots for IRF7 protein expression and ELISA technique for IFN-b1 determination. DMPO-reduced IFN-b1 production and IRF7 expression, whereas increased hemoxygenase-1 expression and restores PPARδ expression. Taken together our results indicate that DMPO prevents LPS-triggered M1-like phenotypic switch of macrophages. Our studies provide critical data for further studies on the possible use of DMPO as a structural platform for the design of novel mechanism-based anti-inflammatory drugs.

Keywords macrophage, lipopolysaccharide, inflammation, phenotypic switch, DMPO

Introduction

Inflammation is an essential and protective response of mammalian cells against irritation. However, a chronic inflammation can lead to tissue damage due to an excessive production of reactive biochemical species (RBS) (oxygen, nitrogen, sulphur and halogen-derived reactive species) and inflammatory mediators (cytokines, chemokines and enzymes) [1]. These mediators are mainly produced by innate immune cells, such as macrophages that acquire an inflammatory phenotype at irritated sites [2].

The most well-known model of inflammatory activation of macrophage is the priming of RAW264.7 cells induced by bacterial lipopolysaccharide (LPS) [1]. LPS triggers a number of transcriptional changes in macrophages causing its inflammatory M1-like switch. These transcriptomic changes can be seen in surface molecular markers such as the co-stimulatory proteins CD80/CD86 [3], CD14 [4] and CD44 [5], together with high levels of inflammatory proteins

such as iNOS, NOX-2, COX-2 [6] and the secretion of cytokines such as Interleukine-1β (IL-1β), type 1 interferons (IFN) and nitric oxide (NO) [6].

Phenotypic plasticity in macrophages are orchestrated by transcription factors that sense the microenviromental stimulus. Among several transcription factors Peroxisome proliferators activated receptors (PPAR δ and γ), Interferon regulatory factors (IRFs) and NF-κB have been pointed as key players in macrophage phenotypic switch [2].

This phenotypic switch is accompanied by an exaggerated production of RBS, which include radical and non-radical species. Reactive oxygen species (ROS), in particular H_2O_2 have been suggested as secondary messengers in redox signaling and inflammatory activation of macrophages [7].

Antioxidants, such as L-ascorbate, urate or glutathione (GSH) can stop free radical reaction throughout becoming itself a free radical by the radical being scavenged, and thus regenerating the native molecule [8]. Unlikely those, spin traps are low-molecular-weight compounds that covalently binds to radical sites in small or large radicalized molecules, and thus can stop free radical-chain reactions that otherwise end in end-oxidation products. Among spin traps, nitrone and nitroxide compounds are the most used in free radical research. Nitrone compounds, such as 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and *N-tert*-butyl-α-phenylnitrone (PBN) offer particular properties that make them suitable for free radical research in cells and organisms. Particularly, DMPO offers interesting properties including low toxicity, known pharmacokinetics, easy diffusion thorough cell membranes and efficient trapping of free radicals [9-11].

By using DMPO as a tool we have studied the intracellular localization and identity of radicalized protein and DNA in cells, tissues and whole animals [12]. Previously we showed that DMPO reacts with radicalized proteins inside the cell and prevents its further oxidation in a model of LPS-induced priming of murine macrophage-like cell line RAW 264.7 [13]. Interestingly DMPO dampened macrophage LPS-driven activation involving nitric oxide (NO) and inflammatory chemokine production by inhibition of NF-κB signaling pathway at early time points after stimulus (*i.e.*, 15 minutes) [14]. These effects cannot be explained by DMPO scavenging ROS, thus interfering in ROS-dependent signaling. The rate constant of DMPO reacting with superoxide is slower than the reaction constant of DMPO reacting with radicalized proteins [15, 16]. These evidences suggest that those protective effects afforded by DMPO on macrophages are not only linked to its spin trapping properties.

Herein we used transcriptomic and proteomic analysis to test whether DMPO causes transcriptional changes that may explain its anti-inflammatory effects in LPS-induced priming of macrophages. This information is critically needed before DMPO or structural analogues proceed to the development of new anti-inflammatory drugs.

Materials and Methods

Cell Culture

RAW 264.7 cells were obtained from American Type Culture Collection (TIB-71, Rockville, MD) and grown in DMEM-F12 medium supplemented with 10% fetal bovine serum (Natocor, Argentina) at 37 °C in a 5% CO_2 incubator. Cells between passages 4 and 12 were used in this study.

LPS and DMPO treatments

LPS (*Escherichia coli* serotype 055:B5, Cat# L2637) was from Sigma (St. Louis, MO). DMPO was from Dojindo Molecular Technologies (Cat# D-04810, Kumamoto, Japan). Cells were cultivated on 25ml T-flasks and allowed to attach for 24 h, then the medium was removed and replaced with the indicated medium with DMPO and/or LPS. DMPO was used at 50 mM in this study. According to our earlier studies, LPS at 1 ng/ml was a non-cytotoxic concentration that primed RAW 264.7 cell to produce significant nitric oxide (NO) [13]. DMPO was used at 50 mM in this study because it could inhibit LPS (1 ng/ml)-induced priming, without significant cytotoxicity as assessed by trypan blue exclusion, lactic-dehydrogenase release and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-reduction assays[13].

NO production assay

Nitrite accumulation in culture medium was measured as described before [14], as an indirect indicator of nitric oxide ($^{\bullet}NO$) synthesis. 10^5 RAW 264.7 cells were seeded on 96-well microplates and stimulated with different

concentration of LPS in the presence or in the absence of 50 mM DMPO for 24 h (final volume 200 µl). After incubation culture medium was collected for nitrite measurement using the Griess reaction. Sodium nitrite was used to produce the standard curve.

RNA isolation

RAW 264.7 cells were incubated in T-75 flask by triplicate with or without 1 ng/ml LPS in the presence or in the absence of 50 mM DMPO. Cells incubated with DMEM culture medium were used as control. After 6 h incubation, cells were washed with PBS, scrapped and pelleted down at 1000*g* for 5´ centrifugation. Resulting pellet was used for RNA extraction using RNAeasy MIDI kit (QUIAGEN). Purified RNA was quantified using NanoDrop® and quality was assessed by using the Bioanalizer® (Agilent, Santa Clara, CA). 50 µg of RNA was then hybridized a Mouse Immunology Codeset (Nanostrings, NS_Immunology_C2269-547 transcripts) as indicated by manufacturer instructions.

Gene expression assay

Gene expression was examined using the NanoString© platform (www.nanostring.com) utilizing Nanostrings Mouse Immunology Codeset (NS_Immunology_C2269) consisting of 547 endogenous and 14 housekeeping genes. 50 ng of each total RNA sample was prepared as per the manufacturer's instructions. Gene expression was quantified on the nCounter Digital Analyzer™ and raw and normalized counts were generated with nSolver (v3.0)™ software. Data were normalized utilizing the manufacturer's positive and negative experimental control probes, as well as the housekeeping gene GAPDH. Results are expressed as mean value ± SEM. Effects were assessed using the Student's *t* test. A difference between treatment groups with $P<0.05$ was considered statistically significant.

Preparation of cell lysates

Following treatments with LPS for the indicated times, RAW 264.7 cell activation was stopped by the removal of medium and addition of ice-cold PBS [17]. In brief, whole cell lysates were prepared and used to detect proteins of interest. Cells were lysed with the CelLytic M lysis solution (Sigma) containing 1% (v/v) protease inhibitor cocktail (Amresco). Cell debris was removed by centrifugation at 12,000 g for 15 min at 4 °C, and the resultant supernatants were stored at −80 °C until use. The protein concentrations in cell lysates were determined using a BCA protein assay kit (Pierce Labs, Rockford, IL) with bovine serum albumin as standard.

Western blot

Cell lysates were mixed with 4 × SDS NuPAGE sample loading buffer (Invitrogen). After heat denaturation, equal amounts of cellular proteins were separated on 4-12% reducing NuPAGE Bis-Tris Gels (Invitrogen), followed by electrotransfer onto a nitrocellulose membrane (0.2 µM pore size). After blocking with 5% non-fat milk in PBS, the immunoblot was performed by incubation with a primary antibody overnight at 4 °C, and then fluorescence goat anti-rabbit or goat anti-mouse IgG secondary antibody for 1 h at room temperature. The immunocomplexes were visualized usingFluorChem HD2 imager (Alpha Innotech Corp., San Leandro, CA). The following primary antibodies were used form ABCAM: IRF7, PPARd and Hemoxigenase-1 (HO-1). Gliceraldehide-phosphate deshydrogenase (GAPDH) from ABCAM was used as loading control.

IFN-β determination

To measure the production of IFN-β cytokine, RAW 264.7 cells were grown 25 ml flasks and treated with LPS and/or DMPO for 24 h. The culture medium was collected and assessed using commercial Mouse IFN-β ELISA kit (N° MIFNB0; R&D Systems; MN; USA).

Statistical analysis

Results are expressed as mean value± s.e.m. Effects were assessed using the Student's t test. A difference between treatment groups with $P<0.05$ was considered statistically significant.

Results

DMPO reduces the expression of surface markers of M1-like phenotypic induced by LPS in RAW 264.7 cells

We previously showed that DMPO decreases LPS-triggered M1-linked pro-inflammatory cytokine (IL-1β, IL-6 and TNF-α) and NO production in RAW 264,7 cells [16]. Herein we were able to reproduce those data by showing that incubation of RAW264.7 cells for 24 h with a low concentration of LPS such as 1 ng/ml was enough to cause significant nitrite accumulation in the culture medium (**Fig 1A**). Furthermore, we show that addition of 50 mM DMPO to the culture medium dampens the LPS-induced nitrite accumulation (**Fig. 1B**).

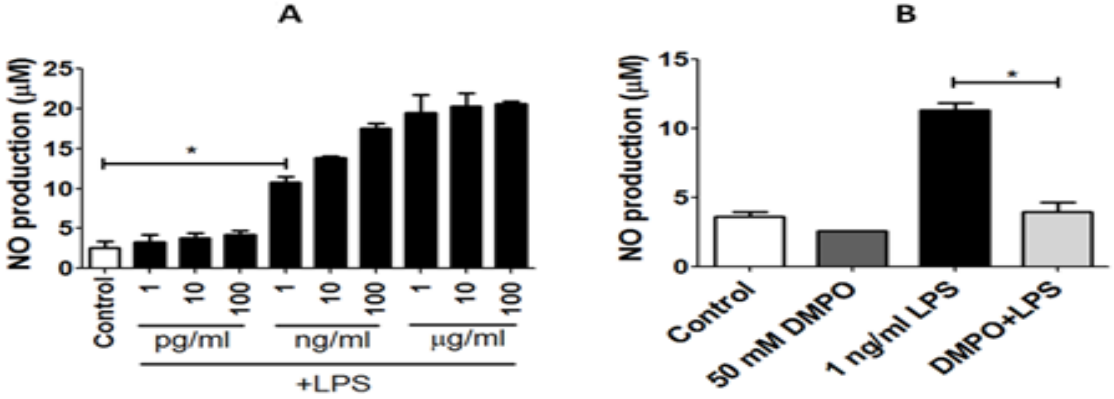

Figure 1: DMPO reduces nitric oxide synthesis in macrophages during priming with LPS

A) Effects of different concentrations of LPS on NO production in RAW 264.7 cells. 10^5 Cells were treated with increasing amount of LPS in a total volume of 200 µl. **B)** RAW 264.7 cells were incubated with 1 ng/ml LPS in the presence or in the absence of 50 mM DMPO. After incubation for 24 h nitrite concentration in the medium was determined using the Griess reaction. Results are shown as mean values ± s.e.m. from 3 independent experiments. Data were analyzed using the **t**-test. *P value < 0,001

Our previous data showed that the reduction of nitrite accumulation in LPS-primed cells caused by DMPO was consistent with low inducible nitric oxide synthase (iNOS) gene expression and reduction of MAPK signaling [16]**, but other markers related to the inflammatory phenotype of macrophages remains undetermined.**

Based on this idea, we decide to measure the expression levels of molecules related to M1-like phenotype of macrophages such as the costimulatory molecules CD80/CD86 [3], the LPS adaptor protein CD14[8], and the cell adhesion molecule related to lymphocyte activation CD44[15] using Nanostring technology (Fig 2).

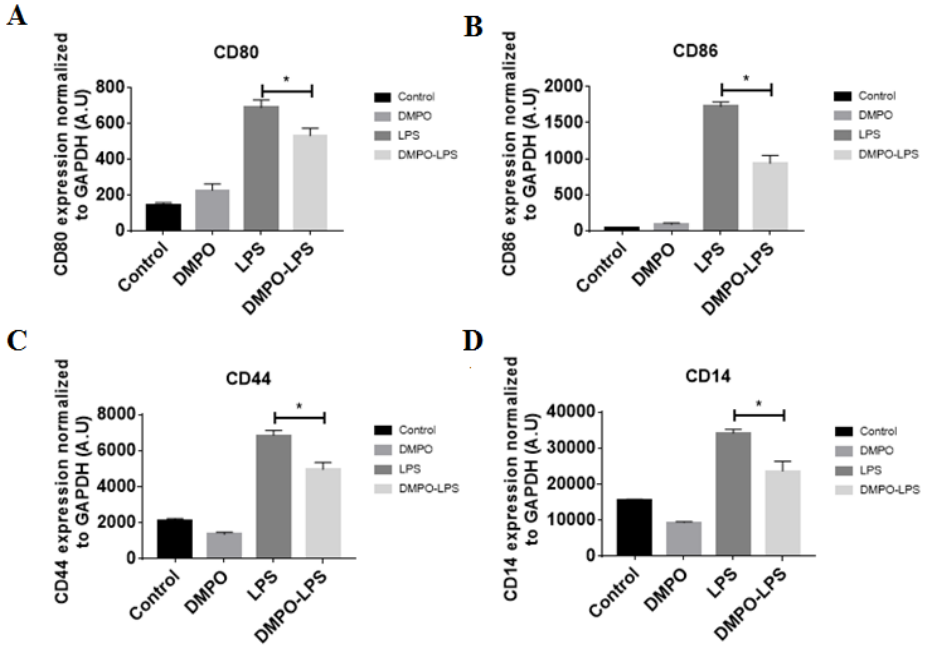

Figure 2: DMPO reduces the expression of surface markers induced by LPS in RAW 264.7 cells

Cells were treated with 1 ng/ml LPS in the presence or in the absence of 50 mM DMPO for 6 h. After incubation gene expression was examined using the NanoString© platform utilizing Nanostrings Mouse Immunology Codeset (NS_Immunology_C2269) consisting of 547 endogenous and 14 housekeeping genes. 50 ng of each total RNA sample was prepared as per the manufacturer's instructions. Gene expression was quantified on the nCounter Digital Analyzer™ and raw and normalized counts were generated with nSolver (v3.0)™ software. Genes were normalized to GAPDH signal. One-way ANOVA test was used to determine statistical differences between conditions. *P < 0.01.

nCounter® results indicate that DMPO is able to reduce significantly the effect of LPS on surface markers of M1-like phenotype. These data is consistent with DMPO affecting several signaling pathways triggered by LPS during macrophage priming.

DMPO blocks interferon signaling triggered by LPS at different levels

Studies have shown that type 1 interferon signaling can be triggered in response to LPS in macrophages among other inflammatory molecules [18]. We have previously shown the effect of DMPO on NF-κB signaling induced by LPS [19] but the effects of DMPO on interferon-related pathways are undefined. Based on this we decided to analyze how DMPO affects interferon signaling at different levels: interferon related transcription factors such as IRF7 by western-blot (Fig. 3C), interferon induced proteins such as Interferon-induced protein with tetratricopeptide repeats 2 (IFIT2) by nCounter® technology (Fig. 3A) and type 1 interferon itself by ELISA technique (Fig. 3B).

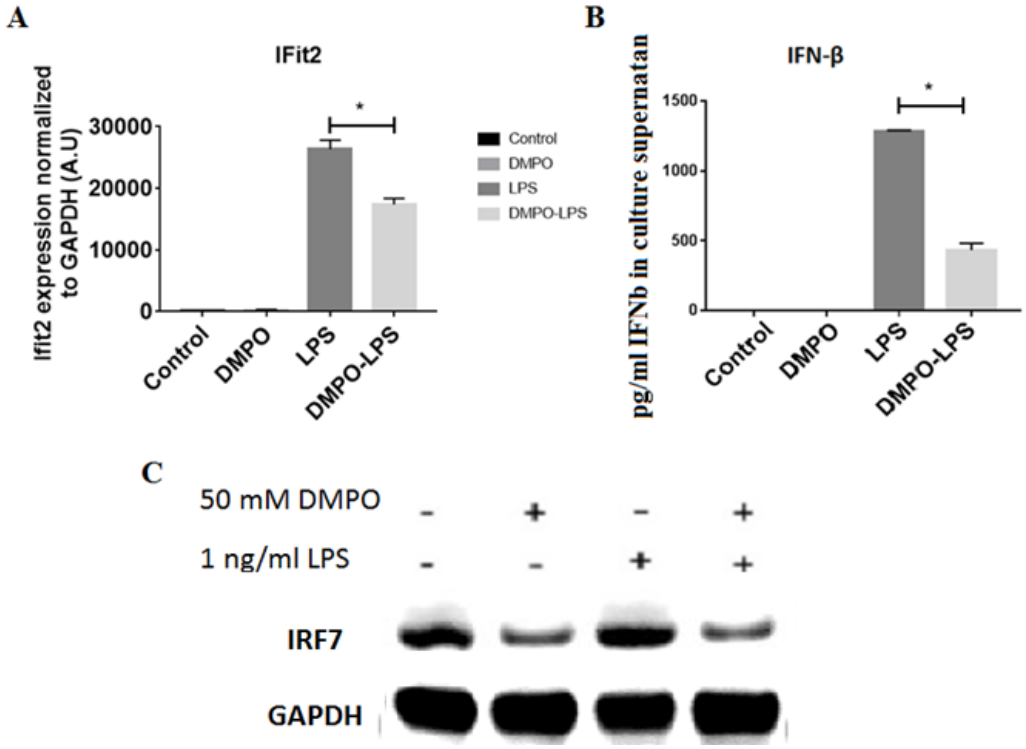

Figure 3: DMPO blocks interferon signaling triggered by LPS at different levels

Effects of DMPO on interferon signaling was analyzed at different levels: (A) nCounter® technology was used for IFit2 mRNA detection after 6 h incubation of RAW 264.7 cells with 1 ng/ml LPS in the presence or absence of 50 mM DMPO. Signal was normalized to GAPDH.; (B) IFN-b concentration measured by ELISA technique on the supernatant of RAW 264.7 cells after 24 hours of 1 ng/ml LPS stimulus in the presence or absence of 50 mM DMPO; (C) IRF7 protein levels determined by western-blot on RAW 264.7 cells incubated with 1 ng/ml LPS in the presence or absence of DMPO for 24 hours. GAPDH was used as loading control. Experiment was carried out by triplicate, most representative picture is shown. * = P< 0,01.

Figure 3 results show that DMPO is able to reduce IRF7 protein levels by itself and in presence of LPS. Moreover, the spin trap Is capable of reducing proteins under the control of IRF7 such as IFN-b and IFIT2. These data clearly shows how DMPO can block the effect of LPS on interferon signaling in murine macrophages.

DMPO restores the expression of PPARδ to control levels in LPS stimulated macrophages and increases the expression of HO-1

Macrophage phenotypic switch is controlled by several transcription factors such as Nf-kb, IRFs and PPARs[2]. PPAR δ has been shown to be a key player in fatty acid metabolism and alternative activation of liver and adipose tissue macrophages [20]. Hemeoxigenase-1 (HO-1) is one of the principle antioxidant proteins expressed alternative activated macrophages among others such as arginase-1 (ARG-1) [21].Following this concept we decided to measure how PPAR δ behaves in LPS stimulated macrophages and which is the effect of DMPO on its expression levels and genes under its control[22]**.** PPAR δ and HO-1 protein expression was measure by western-blot and the results are shown in Figure 4.

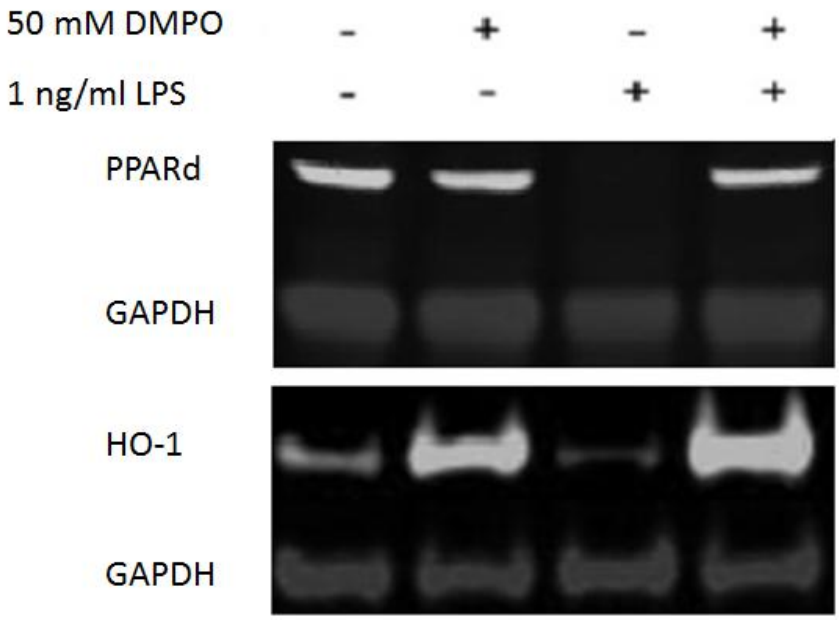

Figure 4: DMPO restores the expression of PPARδ to control levels in LPS stimulated macrophages and increases the expression of HO-1

RAW 264.7 cells were incubated for 24 h with 1 ng/ml LPS in the presence or absence of 50 mM DMPO. After incubation, cells were harvested and proteins were assessed for PPARδ and HO-1 expression by western blot. GAPDH was used as loading control. Experiment was carried out as triplicate and most representative picture is shown.

Results in figure 4 show that DMPO is able to restore the expression levels of PPARδ in LPS-stimulated macrophages. Moreover, DMPO increases the expression of HO-1 by itself and in the presence of LPS. Taking together this results indicate that DMPO not only reduces the expression of M1-like phenotypic markers but also increases the expression of alternative activation markers in macrophages stimulated with LPS.

Discussion

This study shows how DMPO affects the phenotypic switch of macrophages towards a M1-like. We show that the spin trap not only decreases the expression levels of surface molecular markers of classically activated macrophages but also interferes type-1 interferon signaling and restores PPARδ protein levels, a key player in alternative activated macrophage phenotype maintenance.

Nitrone spin traps such as PBN and DMPO were originally developed with the purpose of trapping and stabilizing free radicals, thus making possible their study by ERS or immuno-spin trapping. However, they have shown other properties that made them interesting for pharmacological application as anti-inflammatory drugs. Currently, DMPO or any of its structural analogs have not been moved into the drug development pipeline. On the other hand, PBN-derived compounds have been probed in different models and have reached phase III clinical trials[11]. This situation could be due to DMPO synthesis being really expensive in comparison to PBN and related compounds, and also because transcriptomic effects of DMPO on inflammatory cells remain ill defined. The production of mechanism based–drugs is an emerging field that will lead to safer drugs, especially anti-inflammatory drugs.

PBN and DMPO have been probed to have anti-inflammatory properties on several experimental models. Both spin traps are able to dampen LPS-triggered signaling related to MAPK, Akt, iNOS, COX-2 and pro-inflammatory cytokines [16, 19] suggesting that these effects may be related its nitrone motifs. Moreover, these anti-inflammatory effects cannot be explained by the superoxide scavenging properties of the spin trap due to a low reaction rate constant in both cases[15]. It has been suggested that DMPO reacts with protein and DNA-centered radicals thus interfering the chain reaction for radical production and redox-dependent signaling process [14]. In vivo, DMPO competes with natural anti-oxidant molecules (such as glutathione and L-ascorbate) for free radicals and radicalized macromolecules, thus high doses of the spin trap are needed to overcome the competing reactions [17]. These competing reactions have reactions rate constants much fasters than DMPO, thus formation of nitrone adducts is a kinetically unfeasible process. To the date the exact mechanism by which spin traps exert their anti-inflammatory effect remains unknown.

In this study we showed how DMPO interferes with the effects of LPS on a murine macrophage cell line at the level of surface molecular markers such as CD80 and CD86, both of these molecules are known to be co stimulatory proteins that play a role in setting the immune response when macrophages act as antigen presenting cells[3]. Moreover CD44 and CD14 are famous markers of classical activation of macrophages [5, 23] and both of them were stimulated with LPS and this effect was partially blocked when DMPO is present. We also show how DMPO is able to interfere type-1 interferon signaling by decreasing protein levels of IRF7 and its activity, measure through IFN-b production and IFIT2 protein expression. Furthermore we showed that the spin trap is capable of restoring PPARδ protein levels and increasing HO-1 expression by itself and when LPS is present. These results indicate that DMPO could be switching the phenotype of M1-like LPS-stimulated macrophages towards an alternative activated one. Further studies to define sites of action were DMPO causes these effects are undergoing in order to provide a most robust mechanism of action for this old compound with novel therapeutic properties

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