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Original article

Evaluation of anti-inflammatory and immunoregulatory activities of Stimunex® and Stimunex D3® in human monocytes/macrophages stimulated with LPS or IL-4/IL-13

Cristina Aguzzi [a,1], Oliviero Marinelli [a,1], Laura Zeppa [a], Giorgio Santoni [a], Filippo Maggi [a], Massimo Nabissi [a,b,*]

[a] School of Pharmacy, University of Camerino, Camerino, MC, Italy
[b] Integrative Therapy Discovery Lab, School of Pharmacy, University of Camerino, Camerino, MC, Italy

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ABSTRACT

Macrophages exert an important role in maintaining and/or ameliorating the inflammatory response. They are involved in the activation of an immune response to pathogens, with a balance between the immunomodulatory role and tissue integrity maintenance, however, excessive macrophage activity promotes tissue injury and chronic disease pathogenesis. There is a high interest in evaluating the anti-inflammatory properties of new botanical preparations. Stimunex® and Stimunex D3® are two food supplements formulated as syrups, containing the extract of elderflower (*Sambucus nigra*, Caprifoliaceae), standardized in polyphenol (6%) and anthocyanins (4%), associated with wellmune WGP® β-glucan, with the addiction of vitamin D3 (in Stimunex D3® formulation). The aim of the work was the evaluation of Stimunex® and Stimunex D3® activity in human polarized-macrophages, in order to support their use as supplement for preventing and reducing the inflammatory processes.

In primary human stimulated macrophages, both syrups were able to revert LPS- and IL-4/IL-13-mediated response, reducing the release of several pro-inflammatory cytokines. Results support that these standardized botanical preparations fortified with β-glucan, may have a potential use in the prevention and coadjuvant management of inflammatory process as respiratory recurrent infections and other similar conditions. Moreover, the addition of vitamin D3 revealed to be an advantage in Stimunex D3® for its important role in maintaining and enhancing the innate immune response.

1. Introduction

Macrophages, classified as innate myeloid cells, exert an important role in maintaining and ameliorating the inflammatory response. They are involved in the activation of an immune response to pathogens, with a balance between the immunomodulatory role and tissue integrity maintenance [1]. Based on the microenvironment stimuli, macrophages can polarize in two different types of populations, M1 or M2, which have opposite roles in the inflammatory process [2]. The phenotype M1, or Classically Activated Macrophages, support an inflammatory role. They are stimulated by lipopolysaccharide (LPS) and pro-inflammatory cytokines such as Interferon-γ (INF-γ), Tumor Necrosis Factor-α (TNF-α) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF). These stimulated M1-macrophages release pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, IL-12, IL-23 and TNF-α, resulting in inflammation and promote the antigen-specific Th1- and Th17-mediated inflammatory responses. Instead, M2 or Alternatively Activated Macrophages, are induced by various signals including fungi, parasites, immune complexes, Macrophage Colony-Stimulating Factor (M-CSF), IL-4, IL-13 and Transforming Growth Factor-β (TGF-β). These stimulated M2-macrophages drive the Th2 cell response, especially against parasites, and the release of IL-10, TGF-β and low amount of IL-12, results in anti-inflammatory and immunoregulatory effects. Based on their role to mitigate the inflammatory response, they are usually considered as an

* Corresponding author at: School of Pharmacy, Experimental Medicine Section, University of Camerino, 62032, Camerino, MC, Italy.
E-mail addresses: aguzzicristina02@gmail.com (C. Aguzzi), oliviero.marinelli@unicam.it (O. Marinelli), laura.zeppa.95@gmail.com (L. Zeppa), giorgio.santoni@unicam.it (G. Santoni), filippo.maggi@unicam.it (F. Maggi), massimo.nabissi@unicam.it (M. Nabissi).
[1] Equally contribute.

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anti-inflammatory phenotype, but they can support also an allergic response, driven by Th2 cells [3]. Indeed, increased M2 macrophage polarization and activation has been observed in allergic asthma [4]. The balance in the M1/M2 macrophage polarization plays a pivotal role on destiny of an inflamed or injured organ. M1 polarization is activated when the injury/inflammation/infection is very acute; in this case TNF-α, IL-1β, IL-12 and IL-23 are released in order to activate an inflammatory response. Subsequently, in order to prevent an excessive tissue damage, M2 macrophages, secreting high levels of IL-10 and TGF-β, attenuate the inflammation and allow tissue repair, remodeling and vasculogenesis. In homeostatic condition macrophage activation and polarization are carefully regulated. However, in response to toxicants, this mechanism may be compromised, leading to excessive macrophage activity promoting tissue injury and chronic disease pathogenesis [5].

There is a high interest in evaluating the anti-inflammatory and immunomodulatory properties of new medicinal preparations based on botanicals. These are devoid of possible side effects as those associated with non-steroidal anti-inflammatory therapy [6]. Furthermore, they are well accepted and appealing to consumers.

Among the herbal remedies traditionally used for the treatment of inflammatory and respiratory disorders, the extracts from fruits and flowers of elder (*Sambucus nigra* L.) are much appreciated and exploited for the preparation of phytotherapics and food supplements.

Sambucus nigra is a shrub, up to 4 m tall, belonging to the Caprifoliaceae family and growing in wet woods, clearings, coppice and hedges; it is distributed in central-southern Europe, western Asia and North Africa, from the seal level to 1400 m of altitude [7]. The plant has small, white, pentamer, pot-like flowers grouped in corymbs emitting an aromatic smell; fruits are small drupes of black-purple color. The parts used in the traditional medicines are represented by dry fruits and flowers; the former contain mainly anthocyanosides as cyanidin-3-O-glucoside and cyanidin-3-O-sambubioside, and lectins, whereas the latter are rich in flavonol glycosides (isoquercitroside and rutin) and triterpenes (ursolic acid derivatives and betulin) [8,9].

Stimunex® and Stimunex D3® are two food supplements made up of *S. nigra* extract standardized in polyphenols (6%) and anthocyanins (4%), associated with wellmune WGP® β-glucan and with the addiction of vitamin D3 (Stimunex D3®). The rationale of these formulations relies on the traditional medicine [6]. Actually, it has recently been demonstrated the anti-inflammatory activities of *S. nigra* extract [10–15] as well as the immunostimulant effect of β-glucan [16–25].

β-glucans are natural polysaccharides found in the wall of yeasts, fungi, bacteria, algae, lichens and food plants such as barley and oats. They have immunomodulatory activity and are employed as important ingredients in food supplements, immunostimulants and potential drugs [16]. Glucans are recognized by various receptors, among them Dectin-1, expressed by different immune cells including macrophages, monocytes, dendritic cells and NK cells [16,17,20]. Interacting with macrophages, glucans exert their activity in the stimulated immune system defending it from infections [16].

Vitamin D is an important molecule involved in the organism development and in the maintenance of calcium and bone homeostasis. It has been demonstrated that Vitamin D is able to exert immunomodulatory activity and regulate several molecular pathways of inflammatory process [26,27]. Vitamin D receptors (VDR) expressed in immune cells, such as T and B lymphocytes, monocytes, macrophages and dendritic cells [26], affect the pro-inflammatory e anti-inflammatory cytokines levels and inhibit the Th1- and Th2-specific transcription factors [27].

The aim of the work was the evaluation of Stimunex® and Stimunex D3® activity in polarized-macrophages, in order to support their use as supplement for preventing and reducing the inflammatory processes.

2. Materials and methods

2.1. Reagents

Human Recombinant (hr) GM-CSF (Stem cell Technology), LPS (Sigma Aldrich), IL-4 (Vinci Biochem), IL-13 (VinciBiochem) were dissolved in the respective solvents, aliquoted and stored at −20 °C. Each aliquot was thawed just before use and utilized only once. Stimunex® and Stimunex D3® syrups were kindly provided by Sakura Italia srl. Both syrups are composed by Sambucus standardized in polyphenols (6%) and anthocyanins (4%), wellmune WGP® β-glucan and zinc. Stimunex D3® composition differed only for the presence of vitamin D3.

2.2. Monocyte purification from peripheral blood and Macrophage differentiation

Highly purified $CD14^+$ monocytes were obtained directly from two human whole blood samples, provided by Transfusion center of Macerata Hospital (Italy), by immuno-magnetic negative selection, using the EasySep™ Direct Human Monocyte Isolation Kit (StemCell), following the manufacturing's protocol. Purity assessment of monocytes ($CD14^+CD45^+$) was evaluated by flow cytometry using the following fluorochrome-conjugated antibody clones: Anti-Human CD14 PE- and Anti-Human CD45 FITC-Antibodies (Thermofisher). IgG fluorescent conjugated (FITC and PE) isotypes were used as negative control (Suppl. Fig. 1). Monocytes were cultured in ImmunoCult-SF Macrophage medium (StemCell) and maintained as two separate cell lines named Monocyte population A (MA) and Monocyte population B (MB). As marker for Macrophage differentiation, CD163 detection was evaluated by flow cytometry using Anti-Human CD163 PE-antibody (Thermofisher). IgG fluorescent conjugated (PE) isotype was used as negative control (Suppl. Fig. 2A).

2.3. Sulforhodamine B (SRB) assay

Purified MA and MB cells were seeded at the density of 3×10^4 cells/mL in 96-well plates and treated with 20 ng/mL of M-CSF for 48 hs, to stimulate macrophage differentiation. After treatment, LPS (20 μg/mL), IL-4/IL-13 (10 ng/mL each), Stimunex® (0 up to 10 μL/mL) or Stimunex D3® (0 up to 10 μL/mL), alone or in combination, were added to the wells. Each treatment was performed as repeated administration (after 24 hs from the first treatment). Six replicates were used for each dose. At 48 hs post-treatments, cells were fixed with 10 % cold trichloroacetic acid (TCA) and stained with 0.4 % SRB in 1% acetic acid solution. Unbound dye was removed by washing with 1% acetic acid. Bound stain was subsequently solubilized with Trizma 10 mM. The absorbance of the samples against a background control (medium alone) was measured at 520 nm using an ELISA reader microliter plate (BioTek Instruments, Winooski, VT, USA).

2.4. Gene expression analysis

Total RNA was extracted from MA and MB treated-samples using the SingleShotCell Lysis Kit (Bio Rad) and the evaluation of concentration and quality was performed by spectrometric analysis. Only samples with good quality and concentration were tested by Droplet digital ddPCR. Expressions of IL-1, IL-2, IL-4, IL-6, IL-8, IL-17, TNF-α, GAPDH and β-ACTIN were quantified by ddPCR (BioRad) using pre-designed Assay Primer/Probe Sets, (BioRad). cDNA was generated from each sample on a C1000 Touch thermal cycler (BioRad) starting from 100 ng of mRNA by using iScript Advanced cDNA Synthesis Kit (BioRad,). Briefly, ddPCR was performed using the BioRad QX200 ddPCR system, ddPCRSupermix for Probes (no dUTP), and BioRad standard reagents for droplet generation and reading. End-point PCR with 40 cycles was performed by using C1000Touch Thermal Cycler (BioRad) after splitting each sample into approximately 20,000 droplets. Next, the droplet reader used at least

10,000 droplets to determine the percentage of positive droplets and calculation of copy number of cDNA per GAPDH copy for each sample.

2.5. Cytokines ELISA assay

Cytokines concentration was evaluated in 100 µl of cell culture supernatants from macrophage treated-cell lines by ELISA assay for: IL-1, IL-2, IL-4, IL-6, IL-8, IL-17 and TNF-α. Each sample was analysed after 48 hs post-treatments. Cytokines concentrations were calculated plotting the optical density (O.D.) values in the equation curve obtained with standard, according to the manufacturer's protocol. Each sample was evaluated in duplicated and in two independent experiments.

2.6. Western blot analysis

Cells were treated with vehicle, LPS, Stimunex® and Stimunex D3®, alone and in combination, for 24 h. Lysates were obtained with lisi buffer (composed by TRIS 1 M pH 7.4, NaCl 1 M, EGTA 10 mM, NaF 100 mM, Despxycholate 2%, EDTA 100 mM, TritonX-100 10 %, Glycerol, SDS 10 %, Na2P2O7 1 M, Na3VO4 100 mM, PMSF 100 mM, Cocktail of enzyme inhibitors and H2O). Lysates were separated on a SDS polyacrylamide gel, transferred onto Hybond-C extra membranes (GE Healthcare), blocked with 5% bovine serum albumin in phosphate-buffered saline 0.1 % Tween 20 for 1 h, immunoblotted with rabbit anti-STAT3 (1:2000, Cell Signaling), mouse anti-pSTAT3 (1:1000, Cell Signaling), rabbit anti-iNOS (1:500, Abcam) and rabbit anti-glyceraldehydes-3-phosphate dehydrogenase (GAPDH, 1:6000, Cell Signaling) Abs and then incubated with their respective HRP-conjugated anti-rabbit and anti-mouse (1:2000, Cell Signaling) Abs for 1 h. Peroxidase activity was visualized with the LiteAblot®PLUS or TURBO (EuroClone, Milan, Italy) kit and densitometric analysis was carried out by a Chemidoc using the Quantity One software (Bio- Rad).

2.7. ROS and NO production

Cells at a density of 3×10^4 cells/mL were seeded on a 12 well plate and treatments and vehicle were added. For ROS assay, the fluorescent probe DCFDA was used to assess oxidative stress levels in CCCP-treated cells at 3 h after treatments. Cells were incubated with 20µM DCFDA 20 min prior to the harvest time point. The cells were then washed, and the intensity of the fluorescence was assayed using flow-cytometry and CellQuest software. For NO assay, the cells were treated with LPS (to induce macrophage differentiation) for 24 hs, then the fluorescent probe DAF-FM DA (Sigma Aldrich) at 10 µM was added to evaluate NO production following protocol instruction. The cells were then washed, and the intensity of the fluorescence was assayed using flow-cytometry and CellQuest software (Suppl. Fig. 2C).

2.8. Statistical analysis

Data presented as the mean values ± SE and are representative of the results of at least two independent experiments. The significance of the differences between data (*#$p < 0.05$) was assessed using Student's *t*-test or the one-way analysis of variance (ANOVA). The statistical analysis was performed using Prism 5.0a (Graph Pad).

3. Results

3.1. Stimunex® and Stimunex D3® dose-response in macrophage cell cultures

Stimunex® and Stimunex D3® cytotoxicity was evaluated in MA and MB cells after 48 hs post-treatments, by SRB assay. Both syrups were tested at doses ranging from 0 up to 10 µl/mL and were daily added to the culture medium. The results showed that Stimunex® did not induce cytotoxic effects at doses up to 0.6 µl/mL while Stimunex D3® at doses up to 0.30 µl/mL, in both cell populations (Fig. 1). So, for the next experiments we decided to use one non-cytotoxic dose for each syrup: Stimunex® at 0.3 µl/mL and Stimunex D3® at 0.15 µl/mL.

3.2. Cytokines gene expression analysis

To evaluate the effect of Stimunex® and Stimunex D3® in modulating the inflammatory and immunological response in macrophage populations, both cell populations were treated with LPS or IL-4/IL-13 for 48 hs with daily administration of both syrups. After treatment, cytokines (IL-1, IL-2, IL4, IL-6, IL-8, IL17, TNF-α) gene expression was evaluated by ddPCR. The results evidenced as LPS was able to increase the expression of all cytokines tested except IL-4, and as the treatments with Stimunex® and Stimunex D3® were effective in reducing the LPS-induced cytokines gene expression (Fig. 2 A–F). Similar results were obtained in M2-polarized macrophages stimulated with IL-4/IL-13, since the treatment with IL-4/IL-13 was able to increase the expression of TNF-α, IL-1 and IL-4 and administration of Stimunex® or Stimunex D3® was able to reduce significantly IL-1, IL-4 and TNF-α gene expression (Fig. 3 A–C). Summarizing, these results evidenced as both Stimunex® and Stimunex D3® were able to revert the LPS- and IL-4/IL-13-induced inflammation state in both cell lines population.

3.3. Cytokine release analysis

After the evaluation of Stimunex® and Stimunex D3® in regulating cytokine gene expression, we analysed the LPS- and IL-4/IL-13-induced cytokines content in cell population culture medium, to confirm a correlation between gene expression and protein release. We used the ELISA assay to determine the concentration of cytokines in cell populations culture medium in both LPS and IL-4/IL-13-treated cells in combination or not with Stimunex® and Stimunex D3®. As shown (Figs. 4A-F, and 5 A-C) both syrups were able to reduce the release of all cytokines tested, indicating that the addition of Stimunex® and Stimunex D3® was able to reduce the M1- and M2-polarized responses.

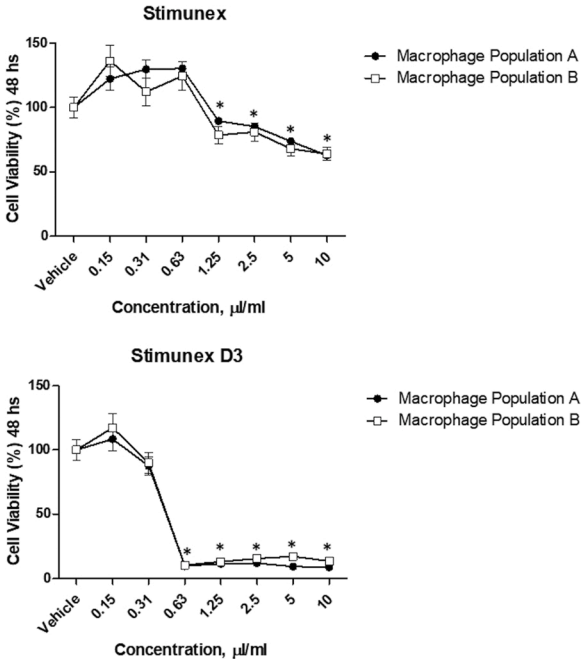


Fig. 1. Effect of Stimunex® and Stimunex D3® on cell viability of two macrophage populations. Populations were treated for 48 hs with different volumes of Stimunex® and Stimunex D3® and cell viability was determined by SRB assay. Data shown are mean ± STD of two independent experiments. *$p < 0.05$ treatments vs. vehicle.

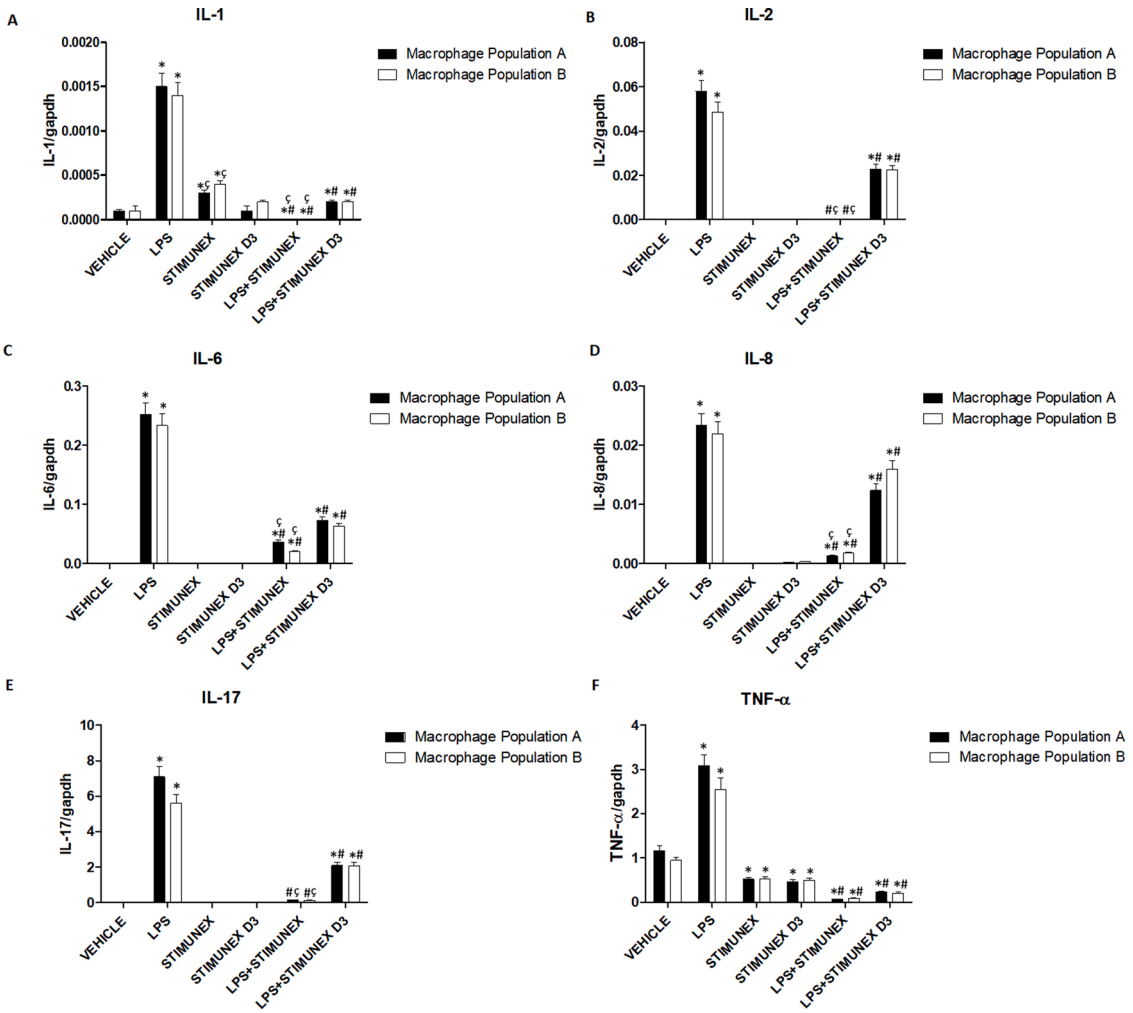


Fig. 2. Effects of Stimunex® and Stimunex D3® on IL-1, IL-2, IL-6, IL-8, IL-17 and TNF-α mRNA levels in M1-polarized macrophages. The expression of IL-1, IL-2, IL-6, IL-8, IL-17 and TNF-α was measured using Droplet digital PCR (ddPCR). Data are normalized to GAPDH. Number of copies was represented as relative quantity. Data shown are mean ± STD of three independent experiments. *p < 0.05 (Student's *t*-test) of treated vs vehicle, #p < 0.05 (Student's *t*-test) LPS + treatments vs LPS, çp < 0.05 (Student's *t*-test) of Stimunex® vs Stimunex D3®.

3.4. Evaluation of Vitamin D3 impact in regulating the macrophage-mediated inflammatory response

Stimunex® and Stimunex D3® are two supplements containing the same ingredients, except for the vitamin D3. Results showed that Stimunex® exerted higher anti-inflammatory activity than the formulation with vitamin D3, mainly in M1-polarized macrophages. Indeed, Stimunex® reduced IL-2, IL-6, IL-8 and IL-17 expression, at both mRNA and protein levels, more than vitamin D_3-containing formulation in M1-polarized macrophages (Figs. 2 and 3 B–E), and in M2-polarized macrophages, Stimunex® reduced IL-4 expression, more than Stimunex D3® (Figs. 4 and 5 C). In addition, the vitamin D3-containing formulation showed higher cytotoxic effect than Stimunex® (Fig. 1).

3.5. Stimunex® and Stimunex D3® reduced ROS production and interfered with STAT-3 LPS-induced activation

Cells at a density of 3×10^4 cells/mL were seeded on a 12 well plate and vehicle, Stimunex® (0,3 μL/mL) and Stimunex D3® (0.15 μL/mL) were added. Results show that after 3 hs Stimunex® and Stimunex D3® reduced ROS production induced by CCCP treatment (Fig. 6). Additionally, after 72 hs of treatments, Stimunex D3® reduced pSTAT-3/STAT-3 ratio, further supporting their role in modulating the inflammatory response (Fig. 7).

4. Discussion

Elderflower and elderberry have traditionally been used to treat different inflammatory conditions, such as sore throat, joint pains, rheumatic pains, paederus dermatitis, but also fever, infections and bites [10–13]. β-Glucans are natural polysaccharides that showed immunomodulatory activity, supporting both the innate and the adaptive immunity [16–25]. Stimunex® and Stimunex D3® are two syrups containing *S. nigra* extract and β-glucan, with the addiction of Vitamin D3 in the second one.

At first, we evaluated the cytotoxic effect of Stimunex® and Stimunex D3® in two different monocytes populations obtained from healthy patients and differentiated in macrophages with M-CSF. Data showed that Stimunex® does not reduce cell viability up to 0.6 μl/mL, while Stimunex D3® at doses up to 0.3 μl/mL after 48 hs of daily treatment. Previous data showed that *S. australis* Cham. & Schltdl. extract had not cytotoxic effect neither in RAW 264.7 macrophages cell line, 3T3 fibroblasts and Hepa 1c1c7 cells at doses from 1.0 μl/mL to 100.0 μl/mL after 24 hs, or in spleen cell culture of normal mice (BALB/c) at doses from 12.5 μl/mL to 100.0 μl/mL after 72 hs post-treatment [11,15]. So far, few data are reported about glucans in vivo and up to now not important cytotoxic effects on normal cells were shown [22]. It has been demonstrated that glucans could be used as anticancer molecules both for their immunomodulatory activity and for their potential

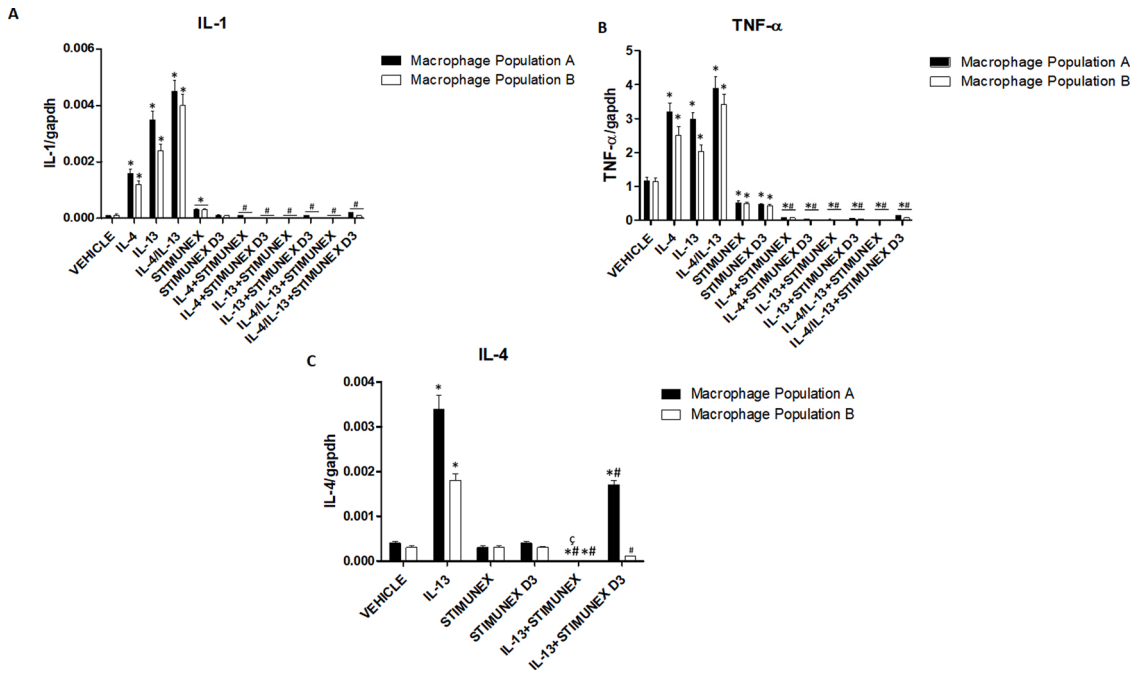


Fig. 3. Effects of Stimunex® and Stimunex D3® on IL-1, IL-4 and TNF-α mRNA levels in M2-polarized macrophages. The expression of IL-1, IL-4 and TNF-α was measured using Droplet digital PCR (ddPCR). Data are normalized to GAPDH. Number of copies was represented as relative quantity. Data shown are mean ± STD of three independent experiments. *p < 0.05 (Student's *t*-test) of treated vs vehicle, #p < 0.05 (Student's *t*-test) LPS + treatments vs LPS, çp < 0.05 (Student's *t*-test) of Stimunex® vs Stimunex D3®.

cytotoxic effect on cancer cells [18,22]. Moreover, β-glucan increased murine macrophage P388/D1cells viability at the doses of 50 and 100 μl/mL, but viability decreased with higher doses, after 24 and 48 hs. Our results demonstrate that Stimunex® was cytotoxic starting from low doses and this evidence can be explained by a potential combination effect between the various formulation ingredients such as *S. nigra* extract and β-glucan.

Vitamin D interacts with VDR receptor expressed on immune cells, including macrophages [27], and studies have demonstrated that vitamin D can inhibit cell proliferation influencing cell cycle, apoptosis and differentiation in a receptor-dependent and independent mechanism [28]. Our results showed that Stimunex D3® induced a higher cytotoxic effect than Stimunex®. This evidence is probably due to a combined activity of Sambucus, glucans and Vitamin D3.

Inflammation is an important component of innate immunity, involving several processes, but an excessive inflammatory response results in tissue damage and chronic diseases [12,13]. On this basis, we evaluated the anti-inflammatory effect of Stimunex® and Stimunex D3® in human stimulated macrophages. M1 macrophages release pro-inflammatory mediators such as IL-1β, IL-6, IL-12, IL-23, TNF-α and inducible Nitric Oxide (NO) synthase [29], while M2 polarization leads to the release of IL-10, TGF-β and IL-12, resulting in anti-inflammatory and immunoregulatory effect [3]. In accordance, our results showed that LPS increased both the expression and the release of IL-1, IL-2, IL-6, IL-8, IL-17 and TNF-α. The treatment of LPS-stimulated macrophages with Stimunex® and Stimunex D3® reverts this effect, with a decrease in the expression and release of all the cytokines cited. Instead, stimulation with IL-4 and IL-13 induces the expression and release of TNF-α, IL-1 and IL-4 and treatment with Stimunex® and Stimunex D3® reverts this effect for IL-1, IL-4 and TNF-α. So far, no data are available about the effect of *S. nigra* extracts in IL-4/IL-13 treated cells, while it has been demonstrated that *S. ebulus* and *S. australis* extracts decreased the release of TNF-α, NO and weakly IL-6, in models of LPS-stimulated murine macrophage RAW 264.7 cells [11,13]. It has been proposed that *S. ebulus* extract exerted its anti-inflammatory activity inhibiting ERK and JNK phosphorylation. Moreover, it decreased the LPS-induced NO production [13]. *S. australis* methanolic extract reverted IL-4, IL-5 and INF-γ expression induced by pokeweed mitogen (PWM) treatment, increased the production of IL-10, and decreased the expression of NF-kB in mice spleen cell culture [11]. Similar results were showed for *S. nigra* aqueous extract on human monocytic THP-1 cells, differentiated in macrophages, and murine macrophages. Indeed, it inhibited the release of TNF-α, inhibiting NF-kB p65 activation and PI3K in both models, but increased IL-10 release only for murine cells [12]. Additionally, *S. nigra* extract reverted the increased IL-6 secretion induced by UVB irradiation in human skin keratinocytes (HaCaTs), as well as the expression of p-JNK, p-p38 and p-ERK. On the contrary, Sambucus promoted the activation of TGF-β/SMAD that was decreased by UVB irradiation [14].

Administration of 1–3, 1–6, D-β-glucan for 14 days in breast cancer patient resulted in increased mean monocyte count in peripheral blood, suggesting that short term oral β-glucan administration could stimulate proliferation and activation of peripheral blood monocytes in vivo [22]. Additionally, particulate β-glucan administered in a mouse model of ovalbumin-induced asthma, inhibited the recruitment of eosinophils and macrophages to the lung, while it promoted a modest increase in neutrophil. Moreover, it reduced Th2 cytokines (IL-4, IL-5, IL-13) production compared to control mice, while Th1 and Th17 cytokine levels were not modulated [24]. Human primary macrophages, treated with 1, 3-β-glucans, showed increased IL-1β production and secretion [19]. Human whole blood was analysed after treatment with soluble branched 1,3-β-glucan alone or in combination with LPS. Glucans induced the release of small but significant amount of TNF-α, IL-6 and IL-8, but the highest dose of glucan was less potent then the lowest dose of LPS. When glucan and LPS were added together, a strong synergistic effect was observed for TNF-α, IL-8, IL-10, but not on IL-6. Instead, pre-incubation with glucan resulted in LPS-induced IL-6 production [25].

Overall, our data showed that Stimunex D3® reverts the inflammatory state mediated by the LPS- and IL-4/IL-13, but to a minor extent than Stimunex®, especially for IL-2, IL-6, IL-8, IL-17 and IL-4. It is well known that Vitamin D3 is involved in regulating the immune response, but it is unclear its role in affecting the cytokines production [30].

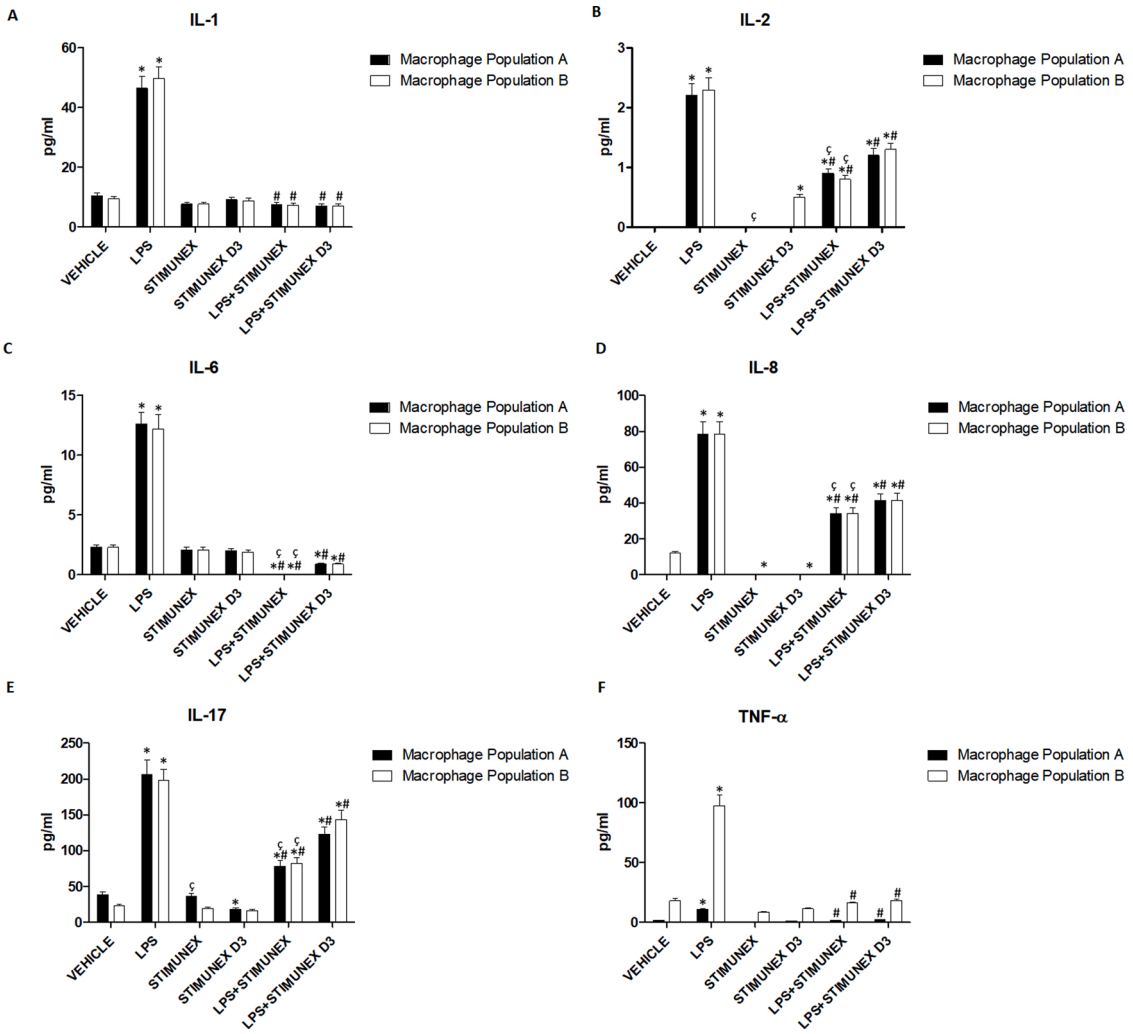


Fig. 4. Effects of Stimunex® and Stimunex D3® on IL-1, IL-2, IL-6, IL-8, IL-17 and TNF-α protein release in M1-polarized macrophages. The release of IL-1, IL-2, IL-6, IL-8, IL-17 and TNF-α proteins was analysed by ELISA in supernatants of two M1-polarized macrophages cultured for 48 hs. Results are expressed as pg/mL. Data shown are mean ± STD of three independent experiments. *p < 0.05 (Student's *t*-test) of treated vs vehicle, #p < 0.05 (Student's *t*-test) LPS + treatments vs LPS, çp < 0.05 (Student's *t*-test) of Stimunex® vs Stimunex D3®.

Indeed, previous studies have demonstrated a dualist behavior on the production of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, INF-γ, TGF-β and TNF-α, in response to a variety of insults [30–32]. Vitamin D inhibited IL-6 and TNF-α levels in LPS-stimulated PBMC cells in a MAPK-dependent manner [33] and reverted LPS-induced HIF-α, IL-1β, IL-6, INF-γ and TNF-α in human oral keratinocyte [34]. Similar results in RAW264.7 macrophage cells in which vitamin D3 treatment resulted also in increased IL-10 and TGF-β levels [35]. Additionally, Vitamin D3 decreased IL-2 and increased IL-4 production in $CD4^+$ $CD25^-$ T cells and PBMC, respectively [31,36]. Opposite results were obtained in macrophages infected by *Mycobacterium tuberculosis* and in LPS-stimulated model, in which the active form of vitamin D3, strongly increased the IL-1β expression, at both mRNA and protein levels. Furthermore, it induced an elevated secretion of other cytokines, including IL-8 and TNF-α, but this was not detected in uninfected cells [30,37]. These evidences suggest that vitamin D3 potentiates the inflammatory innate immune response, without inducing any unwanted inflammatory state, because IL-1β secreted from infected macrophages increases an antimycobacterial response from small airway epithelial cells, leading to a protective effect. These discrepancies could be explained by the fact that in these studies different macrophage phenotypes were used and that vitamin D can both potentiate and inhibit the inflammatory response under different conditions [30]. Furthermore, the anti-inflammatory effect of Stimunex and Stimunex D3 is supported also by the evidence that both syrups were able to revert ROS production Indeed, the excessive ROS production is one of the mechanisms associated with cellular damage in inflammation [38]. In accord with our results, elderberry treatment attenuated UVB-induced ROS production in HaCaT cells [14] and β-glucan treatment attenuated ROS production induced by LPS in human epatocytes [38]. Additionally, we evaluated the effect of syrups on STAT3 activation. It has been demonstrated that STAT3 is involved in pro-inflammatory cytokine-induced inflammatory gene expression and it has a dual role in IL-6 mediated signaling [39]. Both syrups were able to reduce LPS-induced STAT3 activation, mainly were D3 was added, suggesting a regulation of the inflammatory response.

5. Conclusions

In conclusion, our study supports the anti-inflammatory and immunomodulatory effect of a formulation characterized by *S. nigra* extract and β-glucan, which compose Stimunex® and Stimunex D3® supplements, suggesting a potential use of these compositions in the prevention and coadjuvant management of inflammatory process as respiratory recurrent infections and other similar conditions. Moreover, the addition of vitamin D3 revealed to be an advantage in Stimunex D3® for its important role in maintaining and enhancing the innate immune

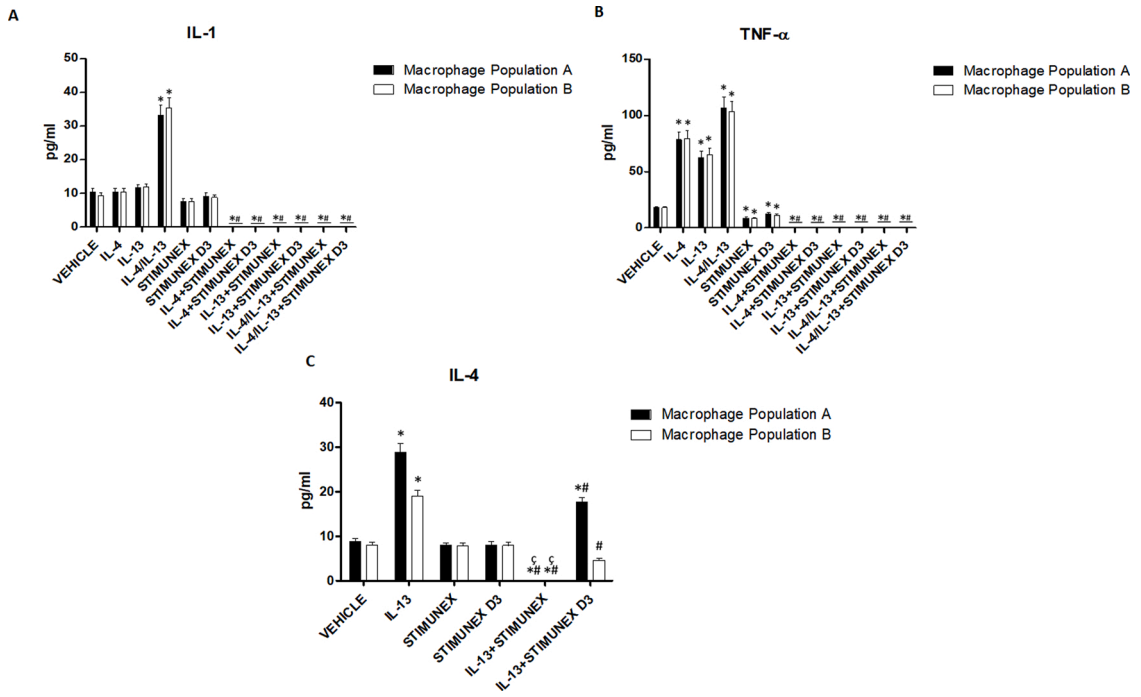
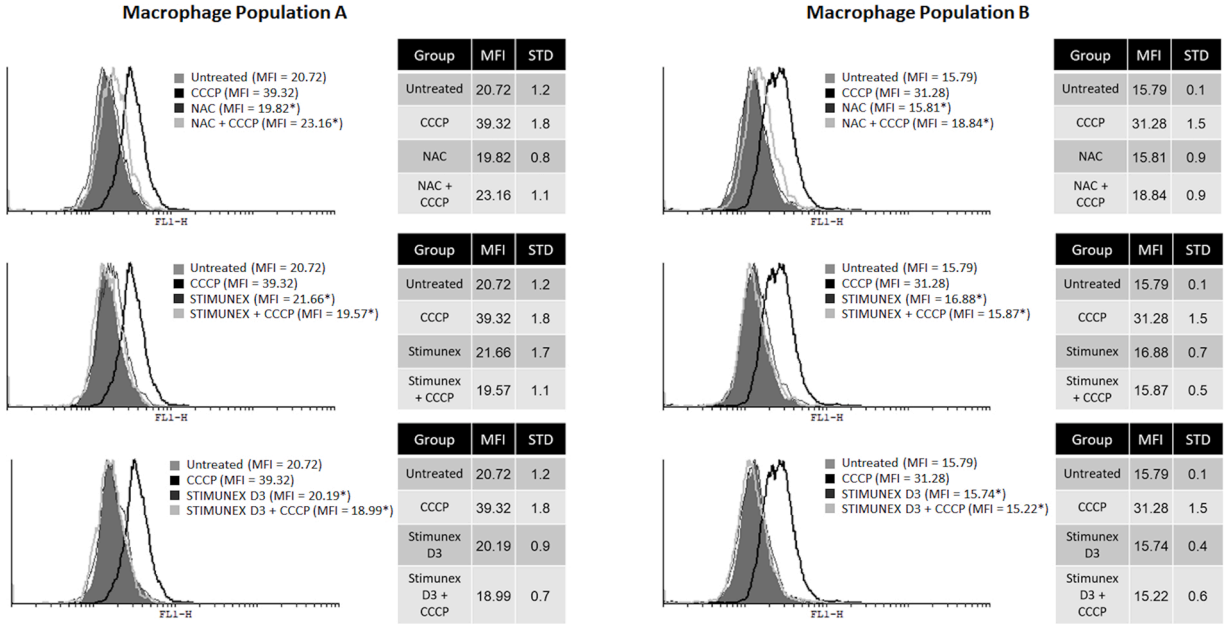


Fig. 5. Effects of Stimunex® and Stimunex D3® on IL-1, IL-4 and TNF-α protein release in M2-polarized macrophages. The release of IL-1, IL-4 and TNF-α cytokines was analysed by ELISA in supernatants of two M2-polarized macrophages cultured for 48 hs. Results are expressed as pg/mL. Data shown are mean ± STD of three independent experiments. *p < 0.05 (Student's *t*-test) of treated vs vehicle, #p < 0.05 (Student's *t*-test) LPS + treatments vs LPS, çp < 0.05 (Student's *t*-test) of Stimunex® vs Stimunex D3®.



Group	MFI	STD
Untreated	20.72	1.2
CCCP	39.32	1.8
NAC	19.82	0.8
NAC + CCCP	23.16	1.1
Stimunex	21.66	1.7
Stimunex + CCCP	19.57	1.1
Stimunex D3	20.19	0.9
Stimunex D3 + CCCP	18.99	0.7

Group	MFI	STD
Untreated	15.79	0.1
CCCP	31.28	1.5
NAC	15.81	0.9
NAC + CCCP	18.84	0.9
Stimunex	16.88	0.7
Stimunex + CCCP	15.87	0.5
Stimunex D3	15.74	0.4
Stimunex D3 + CCCP	15.22	0.6

Fig. 6. Effects of Stimunex® and Stimunex D3® on ROS production in macrophages. The ROS production was analysed by cytofluorimetric analysis of two macrophages population cultured for 3 hs. Cells were treated with NAC, CCCP, Stimunex® or Stimunex D3® alone or in combination. Cells were stained with DCFDA before flow cytometric analysis. Results are expressed as median fluorescence intensity (MFI) with STD values of three independent experiment. *p < 0.05 (Student's *t*-test) of treated vs CCCP.

response.

Authors' contribution

Massimo Nabissi conceived the experiments. Massimo Nabissi, Oliviero Marinelli, Cristina Aguzzi and Laura Zeppa designed the experiments. Oliviero Marinelli, Cristina Aguzzi and Laura Zeppa performed the experiments. Filippo Maggi, Giorgio Santoni contributed for the analysis tool and for writing the paper.

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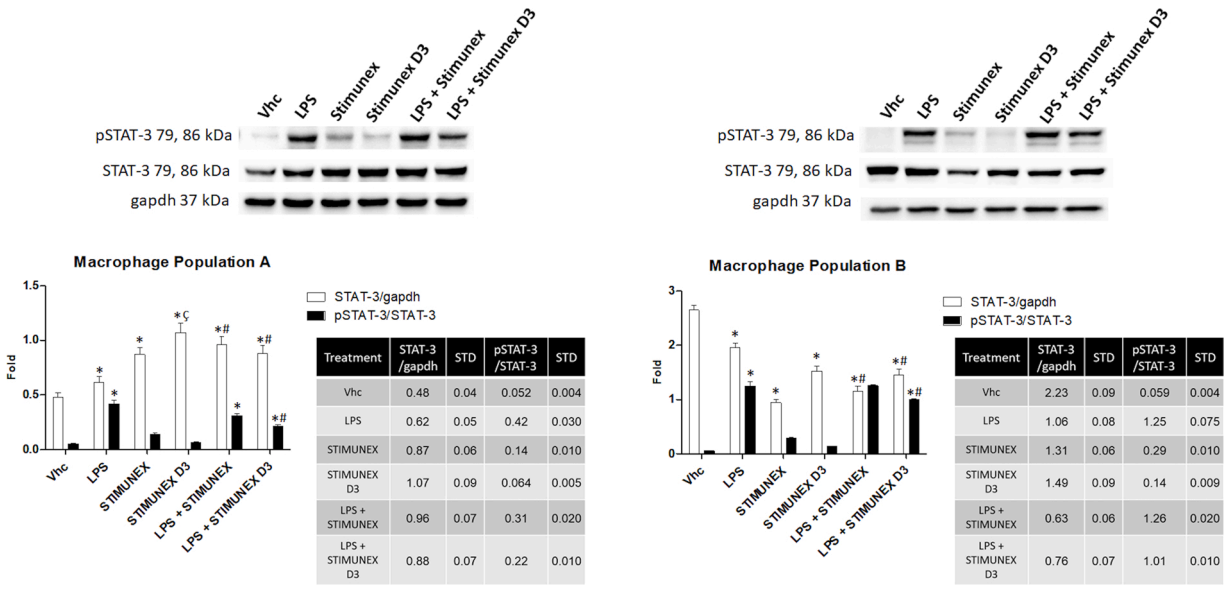

Treatment	STAT-3 /gapdh	STD	pSTAT-3 /STAT-3	STD
Vhc	0.48	0.04	0.052	0.004
LPS	0.62	0.05	0.42	0.030
STIMUNEX	0.87	0.06	0.14	0.010
STIMUNEX D3	1.07	0.09	0.064	0.005
LPS + STIMUNEX	0.96	0.07	0.31	0.020
LPS + STIMUNEX D3	0.88	0.07	0.22	0.010

Treatment	STAT-3 /gapdh	STD	pSTAT-3 /STAT-3	STD
Vhc	2.23	0.09	0.059	0.004
LPS	1.06	0.08	1.25	0.075
STIMUNEX	1.31	0.06	0.29	0.010
STIMUNEX D3	1.49	0.09	0.14	0.009
LPS + STIMUNEX	0.63	0.06	1.26	0.020
LPS + STIMUNEX D3	0.76	0.07	1.01	0.010

Fig. 7. Effects of Stimunex® and Stimunex D3® on STAT3 pathway in macrophages. MA and MB macrophage populations were treated with LPS alone and in combination with Stimunex® or Stimunex D3® for 72 hs. Western blot analysis and densitometric quantification of STAT3 and pSTAT3 protein levels were performed. Densitometric values were normalized to GAPDH used as loading control and shown as mean and STD values. Blots are representative of one of three separate experiments.*$p < 0.05$ (Student's *t*-test) of treated vs vehicle, #$p < 0.05$ (Student's *t*-test) LPS + treatments vs LPS, ç$p < 0.05$ (Student's *t*-test) of Stimunex® vs Stimunex D3®.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgment

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110845.

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