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Scienza e Tecnologia
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UNIVERSITÀ
DI TORINO

Oggetto: **Scientific Agreement between Dept. of Drug Science and Technology and Acfp srl. *Report December 2023***

PURPOSE OF THE WORK

Homeostasis preserves the basic physiological conditions of the human organism, such as body temperature, blood pressure, glucose levels and many other variables. Thanks to millions of biochemical reactions that trigger various biological processes, cells enable all vital organs to function properly. This can only happen in the presence of specific proteins, enzymes and biological cofactors, most of which are already present in the body, but some of which it is important to introduce through proper nutrition.

Macrophages are ubiquitous cells that, playing the role of sentinels, initiate and modulate the immune response. They are one of the body's main transducers and play a key role in preserving homeostasis. They are endowed with plasticity, a peculiarity that makes them capable of perceiving and responding to countless variations in the microenvironment to meet the body's physiological needs. As a result of their plasticity, they can exchange one phenotype for another in response to changes in their microenvironment. This process is called polarisation and allows them, by acquiring a specific functional phenotype, to be able to respond to signals and stimuli from the local microenvironment of a specific tissue (Viola A. et al., 2019; Sica & Mantovani, 2012). Macrophages are classified into two classes:

1. Classically activated macrophages, also referred to as **M_1** (Pace J. L. et al., 1983; Nathan C. F. et al., 1983).
   They are typically pro-inflammatory, secreting high levels of pro-inflammatory cytokines such as TNF-α, IL-1α, IL-1β, IL-6, IL-12, IL-23 and low levels of IL-10.

2. Alternatively activated macrophages, also called **M_2** (Stein M. et al., 1992; Doyle A. G. et al., 1994).
   They have a pronounced anti-inflammatory activity, producing IL-12 to a lesser extent and both IL-10 and TGF-β in large quantities. Functionally, M_2 have a powerful phagocytic capacity because they are able to eliminate debris and apoptotic cells. In this way they are able to attenuate inflammation, promote tissue remodelling, as well as promote and stimulate angiogenesis and immunoregulation.

Since inflammation represents one of the major contributors to the alteration of homeostasis, this work investigates whether the treatment with 22.22 New Cell Code® supplement, studied by the company ACFP srl, may preserve homeostasis by influencing macrophage behaviour in both, basal condition and in the presence of inflammatory stimuli.

Experimental model: human monocyte THP-1 cell line differentiated in macrophages and activated into either M_1 or M_2 macrophages.

The following aspects were evaluated:

- **Toxicity** of 22.22 New Cell Code® supplement:
  - effect on cell growth in THP-1 cells differentiated into macrophages;
  - effect on cell growth in Human umbilical vein endothelial (HUVEC) cells;
- **Activity** of 22.22 New Cell Code® supplement:
  - effect on macrophages migration;
  - effect on LPS-induced expression of cyclooxygenase II;
  - effect on LPS-induced IL-1β release;
  - effect on granulocytes adhesion to HUVEC.

RESULTS

1. EFFECT OF 22.22 New Cell Code® TREATMENT ON CELL GROWTH IN THP-1 CELLS DIFFERENTIATED INTO MACROPHAGES

To assess the potential cytotoxic effect of the 22.22 New Cell Code® supplement, THP-1 cells differentiated in M_0 macrophages (by a 48-hour treatment with phorbol 12-myristate 13-acetate [PMA]) were exposed to either vehicle alone or increasing concentration of the supplement (1:1000, 1:750, 1:500, 1:250, 1:100) for 24 h or 48 h. Cell growth was measured by MTT assay. The results (Figure 1), expressed in percentage compared to untreated cells, show that the product does not induce any significant changes on macrophage growth, thus indicating that it does not exert toxic effects.

A B

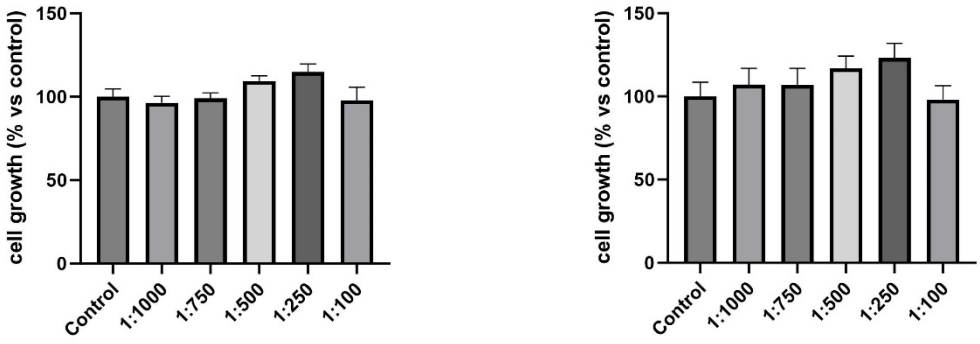

Figure 1 Effect of the supplement on cell growth in differentiated THP-1 cells in macrophages.

Evaluation by MTT assay of M_0 macrophages cell growth after 24 h (panel A) or 48 h (panel B) of supplement treatment. Data are expressed as mean ±SEM of three independent experiments conducted in triplicate.

2. EFFECT OF 22.22 New Cell Code® TREATMENT ON MACROPHAGES MIGRATION

A chemotactic assay (Boyden's assay) was used to evaluate the potential effect of the supplement on the migration ability of M_1 and M_2 cells using CCL7 (30 nM) as chemotactic factor, a chemokine suitable for inducing chemotaxis of both macrophage phenotypes.

Cells were pre-treated with the 22.22 New Cell Code® supplement at different concentrations (1:2000, 1:1000, 1:500 and 1:250) for 24 h. As shown in Figure 2, the treatment induced an increase of M_0 macrophages migration that was significant starting from the concentration of 1:1000. Interestingly, the supplement demonstrated an opposite effect in relation to the phenotype: it reduced the migration of M_1 macrophages (the pro-inflammatory one) and increased the migration of M_2 macrophages. The effect was significant at a dilution of 1:500 for both phenotypes. These results demonstrate a dual anti-inflammatory effect of the treatment that not only reduces the invasion of the inflammatory phenotype M_1 at the level of the lesion, but at the same time it is able to increase the migration of M_2 macrophages, cells that, thanks to the production of anti-inflammatory cytokines and stimulation of remodelling tissue and angiogenesis, have a preponderant role in the resolution of the event (Mantovani et al., 2013).

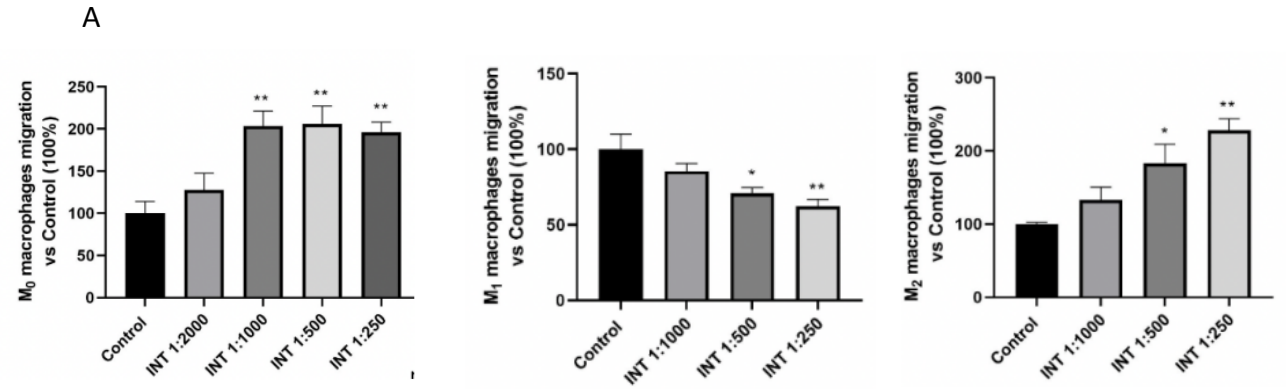

Figure 2 Effect of the supplement on the migratory ability of macrophages.

*The migratory ability of macrophages (M_0, M_1, M_2) treated with CCL7 (30 nM) as chemotactic stimulus in the presence of increasing concentration of the supplement (24 h) was assessed by Boyden's assay. Data are expressed as mean ± SEM of three independent experiments conducted in triplicate. *p < 0.05, ** p<0.01 vs Control.*

3. EFFECT OF 22.22 New Cell Code® TREATMENT ON THE EXPRESSION OF CYCLOOXYGENASE-2 INDUCED BY LPS

In order to further investigate the anti-inflammatory properties of the supplement, a model of acute LPS-induced inflammation was used. In particular, the expression of the pro-inflammatory inducible enzyme COX-2 was assessed by western blot. M_0 macrophages, obtained from PMA-differentiated THP-1 cells, were pre-treated for 24 h with the supplement (1:1000, 1:2000, 1:500) and then for a further 24 h with LPS (100 ng/ml). The analysis performed on the cytosolic extract, obtained after cell lysis, showed that the supplement is able to dose-dependently reduce LPS-induced COX-2 expression. The effect was significant from the concentration of 1:500.

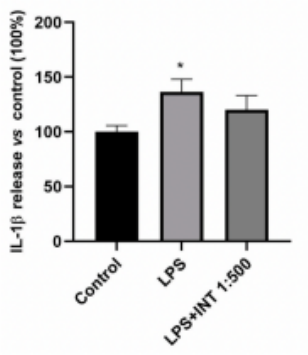

Figure 3. Effect of the supplement on the expression of cyclooxygenase-2 induced by LPS.

*The expression of COX-2 was assessed by western blot in M_0 macrophages. M_0 macrophages were pre-treated with the supplement (24 h) and later with LPS (100 ng/ml, 24 h). Data are expressed as mean ± SEM of three independent experiments conducted in triplicate. *p < 0.05 vs Control; #p < 0.01 vs LPS.*

4. EFFECT OF 22.22 New Cell Code® TREATMENT ON THE RELEASE OF IL-1β INDUCED BY LPS

In order to investigate the potential anti-inflammatory proprieties of the supplement, its effect on LPS-induced IL-1β release was also evaluated. M_0 macrophages, obtained from PMA-differentiated THP-1 cells, were pre-treated for 24 h with the 22.22 New Cell Code® supplement and then for a further 24 h with LPS (100 ng/ml, 24 h). For this experiment, we selected the concentration of supplement that was shown to be effective in inhibiting COX-2 expression, 1:500. Cytokine levels were assessed by E.L.I.S.A. assay performed on the conditioned medium. As can be seen from the Figure 4, a downward trend in IL-1β levels was observed in the cells pre-treated with the supplement (1:500). However, this effect does not reach significance.

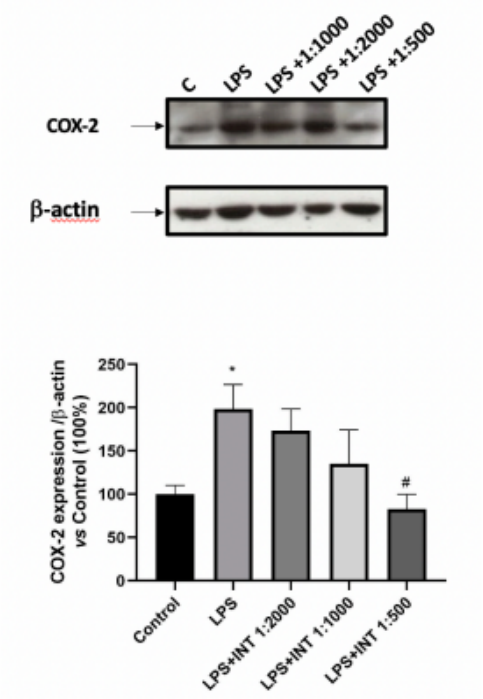

Figure 4. Effect of the supplement on the release of the pro-inflammatory cytokine IL-1β induced by LPS.

*The effect of the supplement on LPS-induced IL-1β release was evaluated by E.L.I.S.A assay. M_0 macrophages were pre-treated with the supplement (1:500, 24 h) and subsequently with LPS (100 ng/ml, 24 h). Data are expressed as mean ± SEM of three independent experiments conducted in triplicate. * p<0.05 vs Control.*

5. EFFECT OF 22.22 New Cell Code® SUPPLEMENT ON CELL GROWTH IN ENDOTHELIAL CELLS ISOLATED FROM HUMAN UMBILICAL CORD (HUVEC).

In order to assess the possible toxic effect of the supplement on HUVEC, the MTT assay was repeated under the same conditions of the previous experiment on THP-1, but increasing the time of the incubation of the cells with the compound: 24, 48 and 72 h of treatment. The results showed that the supplements did not exert toxic effects, except for the highest dose (1:100) that produce a significant decrease in cell growth compared to the untreated cells after 48 or 72 h of treatment.

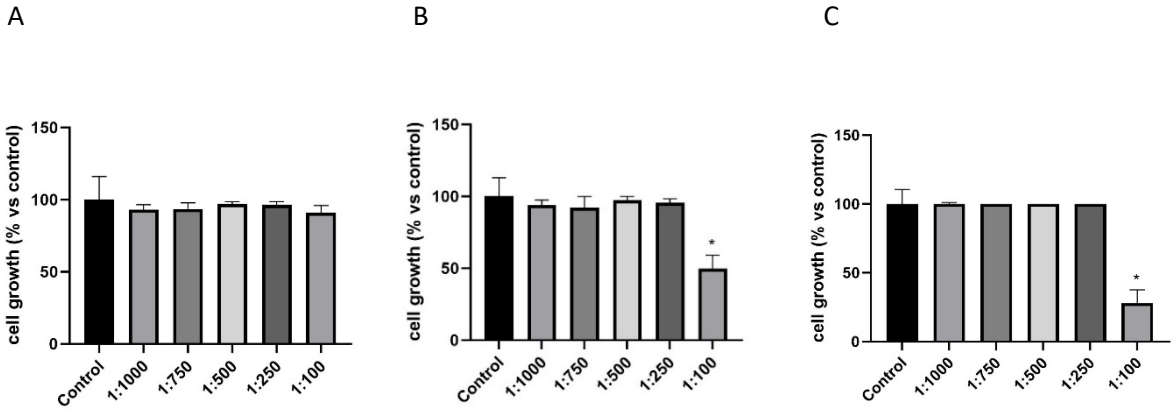

Figure 5. Effect of the supplement on cell growth in HUVEC.

*Evaluation by MTT assay of HUVEC cell growth after 24 h (panel A), 48 h (panel B) or 72 h (panel C) of supplement treatment. Data are expressed as mean ±SEM of three independent experiments conducted in triplicate. * p<0.05 vs Control.*

6. EFFECT OF 22.22 New Cell Code® SUPPLEMENT ON GRANULOCYTE CELL ADHESION TO HUVEC CELLS

One of the initial stages of inflammation is the diapedesis of granulocytes, in particular leukocytes, with which adhesion to the vascular endothelium occurs first through mild selectin-mediated interactions and later through integrins and the stimulation of proinflammatory chemokines. For these reasons, it was evaluated whether the supplement was able to modulate the adhesion of granulocytes to the vessel endothelium. For this purpose, HUVEC cells were pre-treated for 48 hours with different doses of the supplement and TNF-alpha (10 nM), in order to activate the expression of adhesion molecules, and then the adhesion of the granulocytes. The percentage of inhibition against the positive control was assessed: HUVEC pre-treated with TNF-alpha (10 nM). From the results in Figure 6, it is clear that the supplement is able to inhibit granulocyte cell adhesion to HUVECs significantly from the concentration of 1:750.

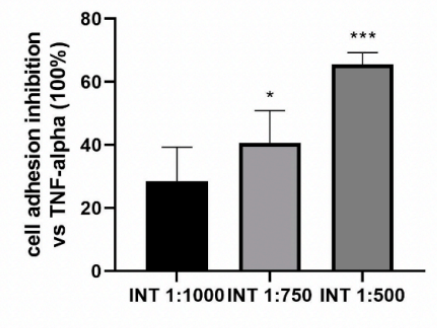

Figure 6. Effect of the supplement on granulocyte cell adhesion to HUVEC. *Evaluation of granulocytes cell adhesion to HUVEC treated with increasing concentrations of supplement (1:1000, 1:750 and 1:500, 48 h) previously stimulated with TNF-α (10nM). Data are expressed as mean ± SEM of three independent experiments conducted in triplicate *p<0.05 vs Control, *** p<0.001 vs Control.*

MATERIALS AND METHODS

Macrophage differentiation

THP-1 cells, were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine 100 µg/ml penicillin-streptomycin and 10% (v/v) foetal bovine serum, at 37 °C in a humidified 5% CO2 atmosphere incubator. Cells were differentiated into macrophages by a 48 h culture with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 48 h, and subsequently in either pro-inflammatory (M_1) or anti-inflammatory (M_2) macrophages by a 24 h culture with either 100 ng/ml lipopolysaccharide (LPS) plus 20 ng/ml interferon γ (IFNγ) or 20 ng/ml interleukin-4 (IL-4) plus 20 ng/ml interleukin-13 (IL-13), respectively.

Measurement of cell viability

THP-1 cells were plated (2×10^4 cells/well) in 96-well culture plates and exposed to PMA for 48 h and subsequently treated with increasing concentration of 22.22 (1:1000, 1:750, 1:500, 1:250, 1:100) for 24 or 48 h. Cell growth was evaluated in sub-confluent cultures by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay; results were confirmed by determining cell density.

HUVEC cells were plated (2×10^4 cells/well) in 96-well culture and subsequently treated with increasing concentration of 22.22 (1:1000, 1:750, 1:500, 1:250, 1:100) for 24, 48 or 72 h. Cell growth was evaluated in sub-confluent cultures by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay; results were confirmed by determining cell density.

Cell motility assay

In the Boyden chamber migration assay, cells (5×10^3 cells/well) differentiated and activated into macrophages M_1 and M_2 were plated onto the apical side of 50 µg/ml Matrigel-coated filters (8.2 mm diameter and 5 µm pore size) in serum-free medium with or without increasing concentrations of the supplement. Nontoxic drug concentrations were used for this assay. Medium containing C–C Motif Chemokine Ligand 7 (CCL7, 30 nM) was placed in the basolateral chamber as a chemo attractant for macrophages. After 6 h, cells on the apical side were wiped off with Q-tips. Cells on the bottom of the filter were stained with crystal violet, and all the fields were counted with an inverted microscope.

Western blot analyses for COX-2 detection

The expression of the inducible enzyme COX-2, a pro-inflammatory maker, was assessed by means of the western blot technique. M_0 macrophages, obtained from PMA-differentiated THP-1 cells, were pre-treated for 24 h with the supplement (1:1000, 1:2000, 1:500) and then for a further 24 h with LPS (100 ng/ml).

About 30 µg of total proteins was loaded for Western blot experiments. After blocking, the PVDF membranes were incubated at 4°C overnight with antibodies against COX-2 (dilution 1:1000). To confirm equal protein loading, membranes were incubated with an anti-β-actin (1:5000) monoclonal antibodies. Proteins of interest were detected with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h at room Temperature.

Measurement of IL-1β concentrations

The effect of 24 h pre-treatment with the supplement on M_0 macrophages on LPS-induced IL-1β release (100 ng/ml, 24 h) was evaluated.

Cell culture supernatants were collected and the levels of IL-1β were quantified with an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions.

Adhesion

HUVEC were grown to confluence in 24-well plates. PMNs (10^7 cells/mL) were labelled with fluorescein diacetate (5 gmL^{-1}) for 30 min at 37°C, washed with BSS, and plated at 10^6 cells/well in a final volume of 0.25 mL BSS on HUVEC pre-treated with the supplement and challenged with different stimuli: TNF- alpha (10 nM). After incubation, non-adherent PMNs were removed by washing three times with 1 mL BSS. The centre of each well was analysed by fluorescence image analysis (Dianzani et al., 2003). Adherent cells were counted employing Image Pro Plus Software for micro-imaging (Media Cybernetics, version 5.0). Single experimental points were assayed in quadruplicate, and standard error of the four replicates was below 10% in all cases. Data are presented as percentage adhesion versus the control value, control adhesion being measured on HUVEC that underwent no treatment. The direct effect on PMNs was assessed by seeding the cells on 24-well EC-free plates for 10–20 min at 37°C, in the presence of the supplement and TNF-alpha. The plates had previously been coated with heat-inactivated calf serum for three hours to reduce spontaneous adhesion to the plastic wells. Percentage inhibition of adhesion was calculated as follows: [100−(a)/(b)]×100, where a is adhesion measured in the presence of the compound plus stimulus minus basal adhesion and b is adhesion elicited by stimulus minus basal adhesion.

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University of Turin - Italy, December 2023