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Combination of metformin with sodium selenite induces a functional phenotypic switch of human GM-CSF monocyte-derived macrophages

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ABSTRACT

Objectives: We evaluated the effects of metformin (Met, 1,1-dimethylbiguanide hydrochloride) combined or not with sodium selenite (Ss, Na_2SeO_3) on the functional activities of LPS-activated GM-CSF monocyte-derived macrophages (GM-MDM).

Materials and methods: Human GM-MDMs from three healthy donors were treated with Met or Ss alone, or with the combination of Met and Ss, and assayed for various biological activities and cytokines expression.

Results: Met alone and Ss alone had significantly different effects on phagocytosis and killing capacities and IL-β production, but had similar effects on the downregulation of inducible nitric oxide synthase (iNOS) activity, relative nicotinamide adenine dinucleotide reduced (NADH) dehydrogenase (Complex I), intracellular free calcium ions ($_{if}Ca^{2+}$), and on the upregulation of arginase activity. Additionally, iNOS activity-to-arginase activity ratio was downregulated in Met or Ss treated-GM-MDMs, and, conversely, upregulated in GM-MDMs treated with Met + Ss in combination, indicating that arginase activity dominates that of iNOS when the two treatments are associated. Moreover, combination of Met with Ss significantly upregulated hydrogen peroxide (H_2O_2) production and phagocytic capacity, but significantly downregulated the production of IL-1β, iNOS activity and killing capacity. On the contrary, we show that Met alone induced significant downregulation of phagocytic capacity and slight upregulation of killing capacity. Nevertheless, Ss seems to accentuate the effect of Met on the downregulation of NO production, as well as to reverse its effect on both phagocytic and killing capacities. On the other hand, all treatments induced a sharp decrease in relative levels of NADH dehydrogenase, and a marked decrease in the levels of $_{if}Ca^{2+}$. Finally, we found that GM-MDMs treated with Met or Ss, or Met combined with Ss exhibited different functional activation phenotypes, as indicated by the surface expression of co-stimulatory and cell activation and presentation molecules CD14, CD80, CD86 and HLA-DR.

Conclusions: Our results demonstrated that Met/Ss combination can play an important role in the modulation of functional activities of human LPS-activated GM-MDMs. Additionally, the overall effects of Met and the induction of "M2" GM-MDMs-associated arginase could be influenced by its combination with Ss.

1. Introduction

Macrophages are differentiated cells of the myeloid lineage that reside in virtually all tissues [1,2]. Considered as the first effectors of the innate immune system [3,4], they act as scavenger cells with high phagocytic functions against external pathogens and apoptotic cell debris [5,6]. They also maintain tissue homeostasis and are professional antigen-presenting cells (APCs). Additionally, macrophages have a significant role in modulating the immune response by regulating inflammatory and apoptotic process, or the adaptive immunity through

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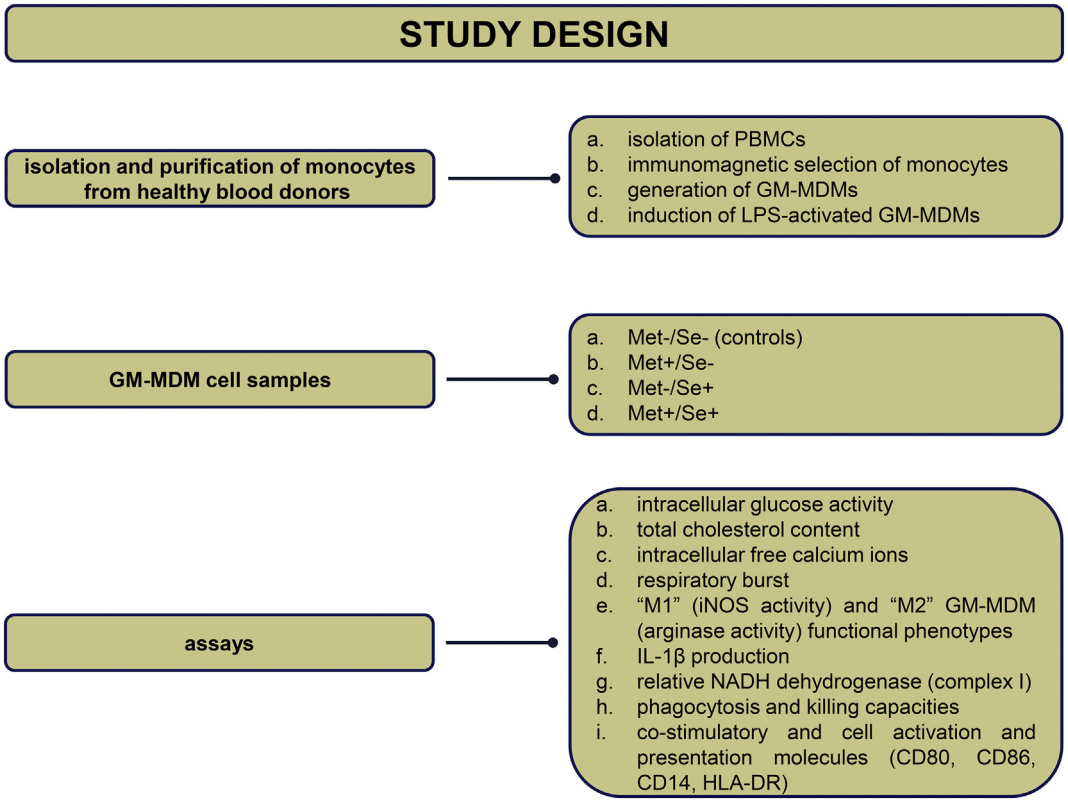


Fig. 1. Study design. This study was conducted in four groups of GM-MDM cell samples, including untreated cell controls (control Met–/Ss–) and cells treated by Met alone (Met + /Ss–) or Ss alone (Met–/Ss +) or Met combined with Ss (Met + /Ss +). Each experiment was performed in duplicate or triplicate and independently repeated at least four times. For flow cytometry, experiments were performed in duplicate and independently repeated three times. Cells were generated from human monocytes, isolated from peripheral blood mononuclear cells by immunomagnetic negative or positive selection, and induced to obtain LPS-activated GM-MDMs. CD14: cluster of differentiation 14, a myeloid differentiation and activation antigen marker of monocyte/macrophage and dendritic cells, which can participate in the response of cells to lipopolysaccharide, CD80/CD86: cluster of differentiation 80/86 (referred to as B7-1/B7-2), family members of B7 (a co-stimulatory signal for T-cell activation), HLA-DR: human leukocyte antigen (class II)–DR, IL-1β: interleukin 1 beta, iNOS: inducible nitric oxide synthase, Met: metformin, GM-MDM: granulocyte-macrophage colony-stimulating factor (GM-CSF) monocyte-derived macrophage, NADH: nicotinamide adenine dinucleotide reduced, PBMCs: peripheral blood mononuclear cell, Ss: sodium selenite.

their abilities to modulate T-cell proliferation and orientation. Moreover, macrophages have a remarkable plasticity to switch from one phenotype to another, that potentially changes the cytokine secretion profile according to the stimuli encountered [7]. These stimuli are various and temporally dynamic leading to the adapted activation and the polarization of macrophages to a specific functional phenotype, also referred to as 'the dichotomous concept'. Activation of macrophages is characterized by the acquired microbicidal, cytocidal and tumoricidal activity. Therefore, activated macrophages have been classified into two major subpopulations, including classically activated, proinflammatory/killer (M1) macrophages or alternatively or anti-inflammatory (M2) macrophages [8,9]. Activated macrophages express high levels of co-stimulatory and antigen presenting molecules, including CD80, CD86 and major histocompatibility complex class I (MHC-I) and MCH-II. Of note, M1 macrophages release a substantial amount of interleukin (IL)-1β and reactive nitrogen species (RNS), mainly nitric oxide (NO); while, activated M2 macrophages upregulate the arginase enzyme [10], which competes with iNOS for L-arginine to produce urea and L-ornithine, polyamines and proline [11]. Therefore, arginase expression is the most significant metabolic marker for M2 macrophages [12]. Increased activation of M2 macrophages is pathogenically linked to abnormal angiogenesis, tumor growth, cell proliferation, and allergic inflammation development [13,14]; whereas, increased activation of M1 macrophages has been associated with autoimmune diseases, in relation to inflammation growth, a strong antimicrobial function, potent cytotoxic and antitumor activity [15].

Metformin (Met, 1,1-dimethylbiguanide hydrochloride) is a biguanid class-drug with antihyperglycemic action, widely clinically used as treatment of type 2 diabetes. It has recently been highlighted for its antitumor and cancer preventive effects [16–18]. Additionally, it has previously been shown that Met can modify macrophage polarization and switch macrophage phenotypes in humans, *in vitro*, when stimulated with lipopolysaccharides (LPS) [19]. Moreover, the potential therapeutic role of Met can be expanded in various conditions, including diabetes, cancer, infections, and several inflammatory diseases [20].

Interestingly, significant attention is given to the combined therapies with Met, especially molecules that have been observed to have an impact on immunomodulation of macrophages and the resolution of the inflammatory responses, like statins and aspirin [21], esomeprazole [22] exenatide and sulfonylureas, dipeptidyl peptidase-4 inhibitors [23], which are tested despite the conventional treatments for cancer, cardiovascular diseases, preeclampsia and diabetes [22,24]. Of note, selenium has shown a great capacity in polarization of macrophages and modulation of the inflammatory status [25], in addition to its low toxicity and side effects when associated to other treatments, including chemotherapy and radiation therapy [26]. Nevertheless, to the best of our knowledge, its association with Met has not yet been explored. Here, we examined for the first time the *ex vivo* effect of the combination of Met with sodium selenite (Ss), a common dietary form of selenium, for its various beneficial effects on immunity [27,28], on functional activities of human LPS-activated granulocyte-macrophage colony-stimulating factor [GM-CSF] monocyte-derived macrophages [MDMs] (GM-MDMs).

2. Materials and methods

2.1. Study design

The effects of Met and Ss were examined on LPS-activated human GM-MDMs, generated from monocytes isolated by immunomagnetic negative selection from peripheral blood mononuclear cells (PBMCs) of three healthy donors without pooling samples. GM-MDM samples were divided into four groups, including untreated cell controls (Met–/Ss–), and cells treated with Met alone (Met + /Ss–), combined (Met + /Ss +) or with Ss alone (Met–/Ss +). Assays were performed on the supernatants or on the whole cell lysates. The levels of NO production, H_2O_2, IL-1β, protein, total cholesterol content ($_{tcc}$CHOL), $_{if}Ca^{2+}$, intracellular glucose ($_i$GLU), relative NADH dehydrogenase (complex I) and arginase activity assays, as well as co-stimulatory and activation and cell presentation molecules (CD80, CD86, CD14, HLA-DR) were carried out on cultures containing GM-MDM cells. Phagocytic and killing capacities were performed on a mixture of GM-MDMs, *Staphylococcus aureus* and Met and/or Ss. Each experiment was repeated independently at least four times and each run in duplicate or triplicate (Fig. 1). This study was approved by the Local Ethics Committee.

2.2. Monocyte isolation and generation of GM-MDMs

PBMCs were isolated from buffy coat blood of healthy donors provided from the "Centre de Transfusion du Sang de CHU Tlemcen" (Algeria) or the "Etablissement Français du Sang" (Montpellier, France) by Ficoll-Paque density gradient centrifugation (Ficoll-Histopaque 1077 Sigma-Aldrich, St Louis, MO, USA). $CD14^+$ monocytes were isolated from PBMCs by negative selection using the Human Enrichment EasySep Monocytes kit (Vancouver, Canada), or by positive selection of $CD14^+$ cells with a magnetically activated cell sorter MACS system (Miltenyi Biotec, Germany), according to the manufacturer's instructions. The cell suspensions purity was higher than 95% as assessed by flow cytometry using the CD14 marker. To generate GM-MDMs, $CD14^+$ monocytes were seeded in 6-well cell culture plates (Thermo scientific, Nunc, Denmark) at 1×10^6 cells/mL in RPMI 1640 medium, 100 µg/mL penicillin, 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS), and 1% nonessential aminoacids (Sigma-Aldrich) supplemented with 50 ng/mL GM-CSF (PeproTech, Rocky Hill, NJ). Cells were incubated for 6–7 days at 37 °C with 5% CO_2, in a humidified incubator. Medium with fresh cytokines was renewed every 2 days [29].

2.3. GM-MDMs culture and treatment

First, both metformin (Met) and selenium in the form of sodium selenite (Ss; Na_2SeO_3) were diluted in purified water to make a 1 M stock solution, and then stored at − 20 °C. GM-MDMs were seeded in 24 or 96 wells (Thermo scientific, Nunc, Denmark) at 2×10^5 cells per well, and activated for 24 h incubation with LPS (*Escherichia coli* 0127:B8, Sigma-Aldrich) [30] at 1 µg/mL [31]. After LPS elimination, matured GM-MDMs were left either untreated (Met–/Ss–) or treated with a dose of 1 mM Met [32–34] (Met + /Ss–) or with 5 ng/mL, *i.e.*, ≅30 nmol/L Ss [27] (Met–/Ss +), or with a mixture of Met combined with Ss (Met + /Ss +), while respecting the same concentrations of each in the final volume of culture medium, in order to obtain *in vitro* responses to treatments, and to avoid cell death or cytotoxicity. Thereafter, cells were incubated for 24 h at 37 °C and 5% CO_2 in RPMI 1640 medium supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, 10% FBS, and 1% nonessential aminoacids.

2.4. Cell lysis and protein assay

Before performing assays for intracellular glucose ($_i$Glu), total cholesterol content ($_{tcc}$CHOL), intracellular free calcium ($_{if}Ca2^+$) and arginase activity measurements tests, the pretreated, treated and untreated GM-MDMs were lysed, as described [35]. Briefly, GM-MDMs were treated with 500 µL 0.1% Triton X 100 for 30 min. To stop the reaction, a mixture containing Tris-HCl and $MnCl_2$ was added. While, for NADH dehydrogenase measurement, GM-MDMs were prepared according to the manufacturer's instructions described in ELISA test kit for NADH dehydrogenase. Intracellular content GM-MDM protein concentrations were assayed spectrophotometrically at 540 nm using a respective kit (Thermo Fisher Scientific Inc., Middletown, USA).

2.5. NO assay, iNOS activity and respiratory burst assay

Cellular generation of NO was determined from the accumulated stable metabolites (nitrite [NO_2-], and nitrate [NO_3-], NOx) in the prior cell culture media. NO levels were measured by the colorimetric Griess assay at 540 nm, using VCl_3, TCA and Griess reagent (Sigma-Aldrich, St Louis, MO, USA), as described [36]. First, 100 µL of supernatants from the GM-MDM culture media were deproteinized with TCA (5%) (Supernatant:TCA, 1:9, v/v). Then, 100 µL of the deproteinized supernatant was seeded into 96-well microtiter plates with 100 µL VCl_3 (8 mg/mL) and 50 µL of Griess reagent. The Absorbance was read at 540 nm using the ELISA plate reader (Biochrom Anthos 2020, Cambridge, UK). After 30 min incubation at 37 °C, concentrations of NOx in supernatants were determined from linear standard curve established by 0–150 µmol/L sodium nitrite ($NaNO_2$). The expression levels of iNOS activity were calculated from NO concentration per mg of proteins per 30 min. Respiratory/oxidative burst assays were performed by measuring levels of NO production and H_2O_2 hydrogen peroxide assays.

H_2O_2 was measured by the adapted method of Pick and Keisari [37] with some variations [30,36,38]. This method consists of the use of a buffered Phenol Red Solution (PRS), which contains a peroxide assay buffer (PAB) (5.0 mM K2HPO4, 1.0 mM KH_2PO_4, 140 mM NaCl, 0.5 mM glucose adjusted to pH 7.4), 0.28 mM (0.1 g/L) of phenol red (phenolsulfonphthalein) and 8.5 U/L (50 µg/mL) of horseradish peroxidase (HRPO, EC 1.11.1.7). The PRS solution was prepared immediately prior to the assay, by adding phenol red and HRPO to 2.1 mL of PAB at a final concentration of 0.46 mM and 0.046 U/mL, respectively. Supernatant were added to the assay mixture at a ratio of 1 to 4 and then incubated for 30 min at 37 °C [30,39]. To stop the reaction, 10 µL of 1 N NaOH was added. The H_2O_2 levels were measured spectrophotometrically at 610 nm against a blank containing buffered PRS and NaOH at the appropriate concentrations. A standard curve was prepared by the use of sequential dilutions of 30% H_2O_2. H_2O_2 concentration was expressed as nmol per 2×10^5 cells per mL.

2.6. Staphylococcus aureus stain

S. aureus ATCC 6538 strain (American Type Culture Collection) was used for the phagocytic and killing capacities' assays. Bacteria were grown in trypticase soy broth medium (TSB) at 37 °C for 18–24 h. The optical density (OD) of 0.5 McFarland turbidity as measured by a colorimeter at 600 nm and quantified against a standard bacterial count corresponded to 10^8 colony forming units/mL. [27,36,40].

2.7. Phagocytic and killing capacities' assays

These assays were assessed as described in detail [36]. The results of phagocytic and killing capacities were evaluated based on percentage of decrease in viable extracellular bacteria and increase in dead intracellular bacteria, respectively. First, GM-MDMs were seeded at 2×10^6/mL per well in tissue culture containing RPMI 1640 culture medium, supplemented with 2 mM L-glutamine and 10% (v/v) FBS, without antibiotics, then subsequently infected with living *S. aureus* at a multiplicity of infection (MOI) of 1:10. At the same time, the same number of bacteria was cultivated alone for use as controls. A viability test of GM-MDMs was made immediately after infection using a

standard procedure (Trypan Blue Exclusion Test). Three hours after incubation at 37 °C in 5% CO_2, supernatants from GM-MDMs containing undigested bacteria, were collected and plated at serial dilutions in sterile PBS on TSB medium then, incubated at 37 °C for 18–24 h. Thereafter, the viable extracellular bacteria were enumerated microbiologically based on the counting of colony forming units (CFUs) to express percentages of phagocytic capacity of both treated and untreated GM-MDMs. Results were calculated as follows:

$$\%\text{phagocytosis} = \text{Mto}–100 \times \frac{\left(\frac{\text{NEC}}{\text{NC1 / NCo}}\right)}{\text{Mto.}}$$

M_{t0}: The number of bacteria in the mixture assay sample at t_0, NEC: the number of extracellular bacteria in the mixture assay sample at t_1, and NC_0 and NC_1 correspond to control samples at t_0 and t_1, respectively.

For the bacterial killing assay, non-phagocytized bacteria were eliminated by washing infected GM-MDMs thrice with approximately 500 µL 1× PBS. Samples were then, incubated for 1 h at 37 °C and 5% CO_2 in culture media supplemented with 50 µg/mL gentamicin for assays with *S. aureus*. To remove remaining antibiotic, cell cultures were washed with 1× PBS and then replaced with RPMI 1640 macrophage cell culture medium. Thereafter, in some wells (W_{to}), 500 µL macrophage lysis buffer was added and incubated for 30 min under a hood with gentle shaking. Other wells were incubated in antibiotic-free medium (W_{t1}) for an additional 1 h and then quenched by addition of macrophage lysis buffer. The cell lysis was stopped by addition of Triton X-100 in TSB. After plating at serial dilutions in sterile PBS on Chapman medium and incubating overnight at 37 °C, we enumerated the viable intracellular bacteria inside the GM-MDMs at time t_o (W_{to}) and at time t_1 (W_{t1}) and calculated the percentage of intracellular bacterial killing as follows:

$$\%\text{bacterial killing} = 100 \times \left(\frac{\text{NWto}-\text{NWt1}}{\text{NWto}}\right)$$

NW_{t0}: the number of viable intracellular bacteria in W_{t0} well at time t_0, and NW_{t1}: the number of viable intracellular bacteria in W_{t1} well at time t_1.

2.8. Arginase activity assay

The activity of arginase (EC 3.5.3.1) was assessed by a spectrophotometric assay based on evaluating the concentration of urea in GM-MDM lysates after the addition of L-arginine [41–43]. Firstly, 25 µL of cell lysates, were inactivated by heating for 10 min at 56 °C, then mixed with 200 µL aliquot of arginine buffer (10 mM L-arg, pH 6.4), and incubated at 37 °C for 1 h. The reaction was stopped by adding acetic acid. The concentration of urea generated after arginine catabolism by arginase was measured at 600 nm using a commercial kit (BioSystems, Spain). Arginase activity was expressed as nanomoles of urea released per mg of proteins per 1 h.

2.9. Relative NADH dehydrogenase (Complex I) assay

To obtain measurements for NADH dehydrogenase in cell lysates, adherent GM-MDMs were initially scraped from the culture well plates and rinsed twice with PBS buffer then solubilized at 2×10^7/mL in extraction buffer. GM-MDMs were incubated on ice for 20 min before centrifugation at 16,000 ×*g* for 20 min at 4 °C. The pellets were discarded and supernatants of cell lysates collected for immediate assay by using a commercial kit for NADH Dehydrogenase Quantification (Complex I) assay (Abcam, UK). The relative NADH levels were spectrophotometrically measured by ELISA procedure after stopping the reaction by addition of 100 µL stop solution (1 N HCl) to each well. Standard curve was established using a preparation of a dilution series from control samples of NADH dehydrogenase (Complex I). All measurements were performed in duplicate. The results were expressed as mg per 2×10^7 cells per mL.

2.10. IL-1β cytokine assay

The concentration of cytokine IL-1β in 24 h culture supernatant of treated and control GM-MDMs was determined by using an IL-1β ELISA kit (Sigma-Aldrich kit), according to the manufacturer's instructions. Absorbance was read at 450 nm in duplicate. The limit of detection (LOD) was 0.3 pg/mL.

2.11. Estimation of $_{tcc}$CHOL levels in GM-MDMs

The quantitative levels of $_{tcc}$CHOL were measured based on an enzymatic spectrophotometric method [44,45] using commercial reagents (Biolabo, France). Cholesterol esters are converted to free cholesterol by the enzyme cholesterol ester hydrolase (EC 3.1.1.1.13). The free cholesterol formed is oxidized to 4 to 3 cholesterol and hydrogen peroxide by cholesterol oxidase and the released H_2O_2 is oxidatively coupled with 4-aminoantipyrin and phenol in the presence of peroxidase. Absorbance was measured at 492 nm.

2.12. $_i$Glu levels assay

$_i$Glu concentrations were spectrophotometrically assessed on supernatants of the GM-MDM lysates using a commercial assay kit (CHRONOLAB Systems, Spain), according to the manufacturer's instructions. This process is based on the oxidation of glucose to gluconate by the glucose oxidase enzyme (GOD), and the H_2O_2 produced was detected at 505 nm [46] with chromogenic oxygen acceptor, phenol, 4-aminophenazone (4-AP) in the presence of peroxidase (POD). The results were expressed as nanomole per mg protein.

2.13. $_{if}Ca^{2+}$ levels assay

$_{if}Ca^{2+}$ levels were assessed on 2×10^5 cells/mL of GM-MDM lysates. The assay was performed by a colorimetric method using a respective kit (Sigma-Aldrich, St. Louis, Missouri, USA). Calcium levels were measured on microplate ELISA reader (Biochrom Anthos 2020, Cambridge, UK). The free calcium contained in supernatants of cell lysates reacted with methylthymol blue in alkaline medium forming a coloured complex that was measured by spectrophotometry at 492 nm. Hydroxyquinoline was added to avoid magnesium interference. The concentrations of calcium were calculated using the formula: [Absorbance (assay) / absorbance (standard)] × Standard concentration × Dilution factor. The results were expressed as µEq per mg protein.

2.14. Flow cytometry assay

The expression levels of CD14, CD80, CD86 and HLA-DR on LPS-activated GM-MDM cells were evaluated by flow cytometry. Antibodies were obtained from BD Pharmingen (San Diego, CA), including FITC–conjugated anti-CD80-antibodies (L307.4), allophycocyanin -(APC)–conjugated anti-CD86 antibodies (2331 FUN-1), phycoerythrin (PE)–conjugated anti HLA-DR antibodies (G866) and phycoerythrin (PE)–conjugated anti-CD14 antibodies (M5E2). After staining, cells were washed 3 times in PBS + 10 SVF + 5 mM EDTA (FACS buffer) and resuspended in FACS buffer. Flow cytometry experiments were performed in duplicate a minimum of three independent times. Results are expressed as mean with SEM unless otherwise stated. Cells suspension (minimum 10^4 gated events per sample) was analyzed using a FacsCalibur flow cytometer (Becton-Dickinson) and FlowJo software (Tree Star Inc., Ashland OR).

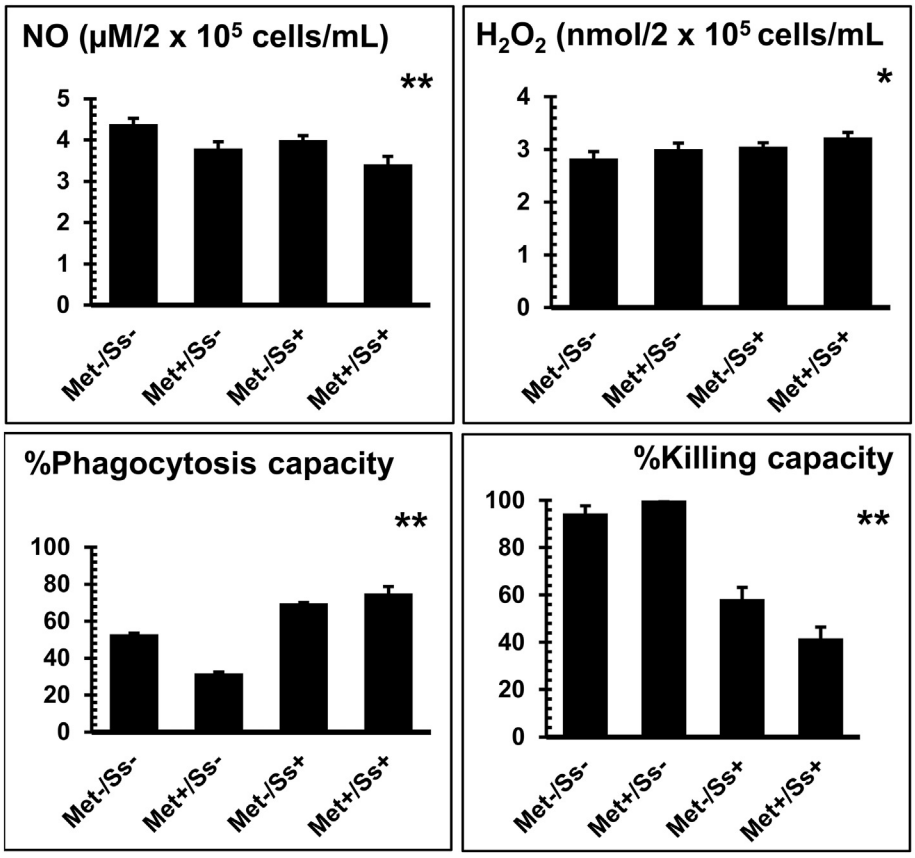


Fig. 2. Effect of Met and Ss on functional activities of GM-MDM. Functional activities of GM-MDM were determined by respiratory burst assays, phagocytic and killing capacities. The respiratory burst (also referred to as oxidative burst) was achieved by measurements of NO production and H_2O_2. *$p < 0.05$, **$p < 0.01$ by Kruskal–Wallis test with pairwise comparisons using the Dunn-Bonferroni approach. The results are presented as mean values with standard error of mean (SEM) of five independent experiments carried out on cell samples isolated from three blood donors ($n = 15$ in each group). H_2O_2: hydrogen peroxide, NO: nitric oxide, Met: metformin, GM-MDM: granulocyte-macrophage colony-stimulating factor (GM-CSF) monocyte-derived macrophage, Met–/Ss–: untreated GM-MDM cell controls, Met + /Ss–: GM-MDM cells treated with metformin alone, Met–/Ss + : GM-MDM cells treated with sodium selenite alone, Met + /Ss + : GM-MDM cells treated with both metformin and sodium selenite, Ss: sodium selenite.

2.15. Statistical analyses

The values are presented as the mean with standard error of mean (SEM). Statistical analyses were performed by a non-parametric Mann-Whitney *U* or Kruskal–Wallis one-way analysis of variance (ANOVA) test, with pairwise comparisons using the Dunn-Bonferroni approach, as the data were not normally distributed [47–49]. Data were analyzed using SPSS software package version 20.0 for Windows (SPSS Inc., Chicago, IL, USA), and differences were considered statistically significant at *p*-value < 0.05.

3. Results and discussion

3.1. Effect of Met and Ss on functional activities of GM-MDM

The results in Fig. 2 show that H_2O_2 production was significantly increased in GM-MDMs treated with combined Met + /Ss + compared to untreated controls ($p < 0.05$). Additionally, we show that both Met alone and Met + /Ss + significantly downregulated the levels of GM-MDMs NO production in comparison to untreated controls (respectively, $p < 0.05$ and $p < 0.01$). Moreover, a significant decrease of NO levels was observed in Met + /Ss + in comparison to Ss treatment alone ($p < 0.05$). On the other hand, phagocytic capacity levels were significantly increased when comparing Ss and Met + /Ss + treated-GM-MDM with Met and Ss untreated-GM-MDM control cells, but they were significantly decreased in Met treated-GM-MDM. In contrast to the phagocytic capacity, killing capacity was slightly upregulated in Met treated-GM-MDM, and, conversely, was significantly downregulated in Ss or Met + /Ss + treated-GM-MDM (for all comparisons, $p < 0.01$). Therefore, Ss seems to accentuate the effect of Met on NO production, but appears to reverse its effect on phagocytic and killing capacities.

Macrophages are versatile innate immune cells endowed with a crucial ability for keeping a balanced response to environmental homeostatic signals [2,50]. They are characterized by their high functional plasticity and capacity to be "re-polarized" or "re-programed" into M2 or M1 phenotypes through exposure to specific micro-environmental signals, but also to bioactive molecules and drugs. When this delicate balance is disturbed, inappropriate activation can take place. This phenomenon may lead to an alteration in homeostasis and an inflammatory state, and could even generate positive changes in their function and active metabolism.

During respiratory burst, activated macrophages, especially M1 macrophages, produce reactive oxygen (ROS) and nitrogen (RNS) species following phagocytosis. Interestingly, the evaluation of H_2O_2 and NO production, as well as phagocytic and killing capacities should be sufficient to attest the course of the *ex vivo* macrophage activation and changes in functional activities. In our study, we observed that Met or Ss treatment in one case induced upregulation of H_2O_2 production and phagocytic capacity, and, on contrary, induced downregulation of NO production or killing capacity. Accordingly, recent studies demonstrated that both Met and Ss have a major metabolism immunomodulatory activity on macrophages [18,51,52]. Met is supposed to attenuate the inflammatory feedback loop, especially in cancer cells, and has been shown to inhibit iNOS expression [17]. Likewise, selenium was reported to play critical roles in anti-inflammatory activities [53], probably by its capacity to downregulate the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB)-dependent signaling pathways [25] and the expression of proinflammatory genes and mediators. Similar to Met, selenium can also downregulate the activity of iNOS following stimulation with LPS [51], which corroborate with our findings. Moreover, it has been reported that selenium supplementation can also play a key role in M1/M2 switching process and modulate the polarization of proinflammatory activated M1 macrophage toward anti-inflammatory, alternatively activated M2 macrophage [54]. Of note, we have recently shown that a low dose of Ss induced a significant decrease in NO production by intraperitoneal

macrophages, but a significant increase in H_2O_2, which could be explained by the significant impact of Ss to regulate the release of free radicals [27]. Finally, the macrophage phagocytic activity has been reported to be a functional marker not only for proinflammatory, classically activated M1 macrophages, but also be associated with a typical M2-like macrophages that more specifically and efficiently phagocytose and engulf apoptotic cells [55]. As we see in the current study, the combined effects of Met and Ss have an impact role on all functional activities of GM-MDM, including respiratory burst and phagocytic and killing capacities. Of note, we observed that Met + Se induced downregulation of both killing capacity and NO production, suggesting that NO may exert a more important effect on the lysis of the bacteria used in the experiment, *i.e.*, *S. aureus*, than H_2O_2 in human GM-MDMs. Finally, Ss seems to have a dominant effect on Met, given that for phagocytosis, the enhancing effect is due to Ss and seems that Ss can revert the inhibitory effect of Met and that the combination of Ss with Met further induced a decrease in killing capacity, whereas that Met alone slightly increased this activity.

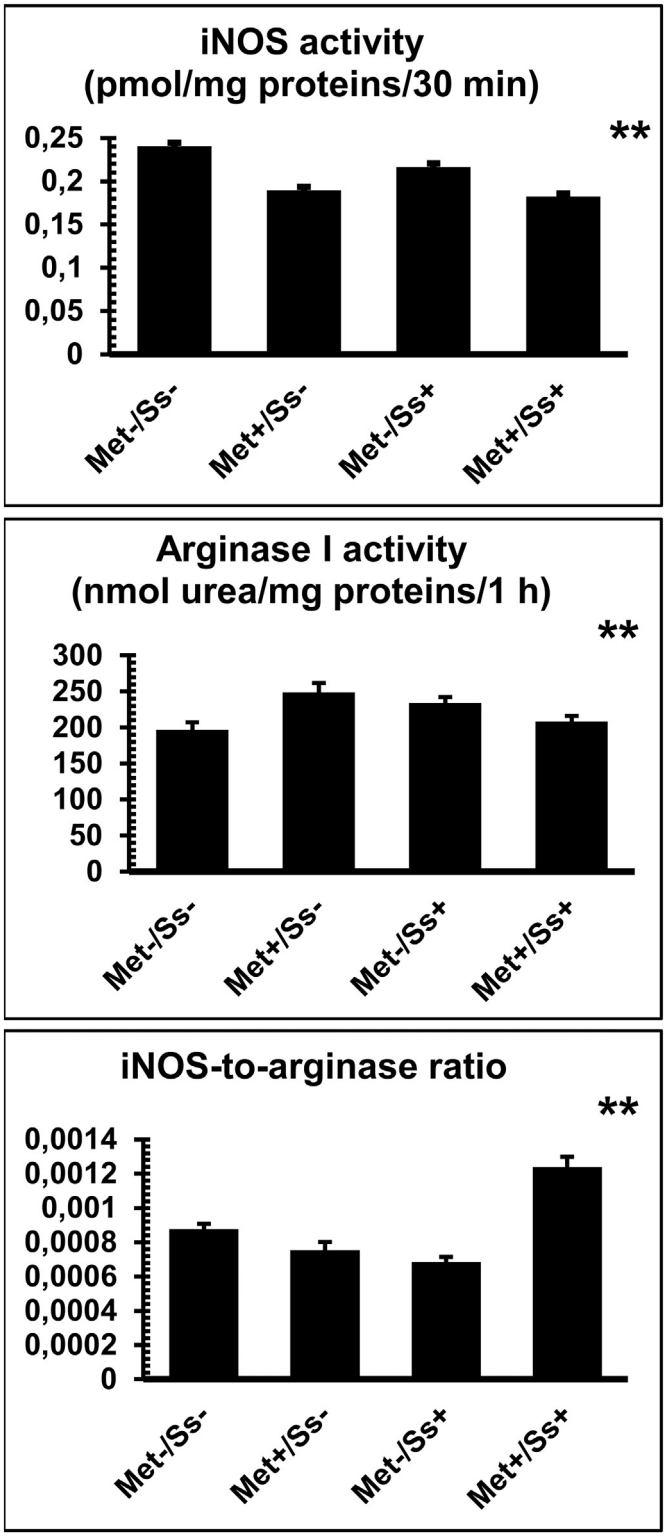
3.2. Effect of Met and Ss on GM-MDM iNOS and arginase activity and iNOS activity-to-arginase activity ratio

As shown in Fig. 3, results revealed that both Met and Ss treatments significantly downregulated the levels of iNOS activity in comparison to untreated controls (for all comparisons, $p < 0.01$). Conversely, arginase activity was significantly increased in Met or Ss treated-GM-MDMs when compared to untreated controls ($p < 0.05$ by Mann-Whitney *U*). Additionally, treatment with Met alone induced a significant increase in arginase activity in comparison to combined Met and Ss ($p < 0.05$ by Mann-Whitney *U*). Moreover, the ratio of iNOS activity-to-arginase activity decreased significantly in GM-MDMs treated with Met or Ss alone in comparison with untreated controls (for both Met and Ss, $p < 0.05$), or with combined Met + Ss ($p < 0.001$ by Mann-Whitney *U* test). *p*-Values with Kruskal-Wallis tests were < 0.01 for all comparisons.

In our study, we demonstrated that treatment with both Met and Ss results in upregulation of arginase activity and downregulation of iNOS activity and NO production. Given that M2 macrophage cells are commonly categorized by the enzyme arginase, the competition between arginase and iNOS for the aminoacid L-arginine substrate has been recognized as an effective way of inducing downregulation of NO production [10], and leading to the M2 macrophage polarization [10,51]. Accordingly, our recent results [27] have demonstrated that Ss induced a significant increase in the macrophage arginase activity. Moreover, it has been reported that selenium downregulated proinflammatory cytokines, like IL-1β, and iNOS following stimulation with LPS, and upregulated the levels of anti-inflammatory biomarkers, including arginase I [51,56], which is in accordance with our results. Potentially, this could be due to the modification of the arachidonic acid pathway [57] through the modulation of key transcription factors that mediate the activity of selenium, including signal transducer and activator of transcription 6 (STAT6) and peroxisome proliferator-activated receptor gamma (PPARγ)-dependent pathways [51]. In addition, it has previously been reported that Met raised arginase I protein expression, which coincides to the downregulation of NO production and attenuation of iNOS expression [58]. Mechanistically, the inhibition of iNOS activity and NO production would be related to the activation of 5′ adenosine monophosphate-activated protein kinase (AMPK) signaling pathway [18] following treatment with Met, as reported in murine macrophages [59]. Furthermore, similar results have shown that Met can inhibit LPS-induced NO production and iNOS expression in mouse macrophage-like cell line RAW2647 [60].

3.3. Effect of Met and Ss on the production of IL-1β by GM-MDM

As indicated in Fig. 4, the production levels of IL-1β were



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significantly increased in Ss treated GM-MDM cells in comparison with untreated GM-MDM controls ($p < 0.01$); conversely, a significant decrease of IL-1β levels was observed with combined Met + /Ss + treatment when compared to controls ($p < 0.05$). In addition, treatment with Ss alone demonstrated a significant increase in the production of IL-1β in comparison to the cells treated with combined Met + /Ss + and with those treated with Met alone (respectively, $p < 0.001$ and $p < 0.01$ by Mann-Whitney *U* test). Kruskal-Wallis tests gave a *p*-

Fig. 3. Effect of Met and Ss on GM-MDM iNOS and arginase activity and iNOS activity-to-arginase activity ratio. Arginase activity levels were determined spectrophotometrically by measurement of the urea concentration after the addition of L-arginine. The iNOS activity was determined from NO levels relative to protein concentration. $^{**}p < 0.01$ by Kruskal–Wallis test with pairwise comparisons using the Dunn-Bonferroni approach. The results are presented as mean values with standard error of mean (SEM) of five independent experiments carried out on cell samples isolated from three blood donors ($n = 15$ in each group). Met: metformin, Ss: sodium selenite, GM-MDM: granulocyte-macrophage colony-stimulating factor (GM-CSF) monocyte-derived macrophage, iNOS: inducible nitric oxide synthase, Met–/Ss–: untreated GM-MDM cell controls, Met + /Ss–: GM-MDM cells treated with metformin alone, Met–/Ss + : GM-MDM cells treated with sodium selenite alone, Met + /Ss + : GM-MDM cells treated with both metformin and sodium selenite.

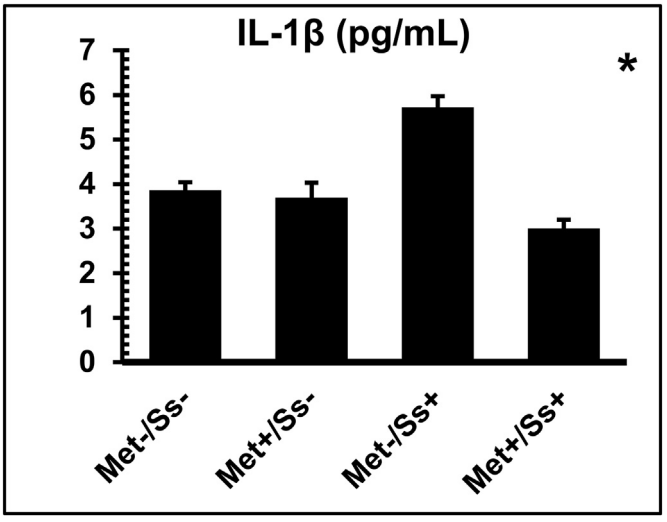

Fig. 4. Effect of Met and Ss on GM-MDMs IL-1β production. IL-1β levels were measured using sandwich enzyme-linked immunosorbent assay (ELISA). $^{*}p < 0.05$ by Kruskal–Wallis test with pairwise comparisons using the Dunn-Bonferroni approach. The results are presented as mean values with standard error of mean (SEM) of four independent experiments carried out on cell samples isolated from three blood donors ($n = 12$ in each group). IL-1β: interleukin 1 beta, Met: metformin, Ss: sodium selenite, GM-MDM: granulocyte-macrophage colony-stimulating factor (GM-CSF) monocyte-derived macrophage, Met–/Ss–: untreated GM-MDM cell controls, Met + /Ss–: GM-MDM cells treated with metformin alone, Met–/Ss + : GM-MDM cells treated with sodium selenite alone, Met + /Ss + : GM-MDM cells treated with both metformin and sodium selenite.

value < 0.05 when comparing all GM-MDM groups.

IL-1β is a highly potent proinflammatory cytokine produced by diverse cell lines, mainly macrophage cells [61,62]. It is considered as a key cytokine secreted upon classical activation of M1 macrophages [30]. Activation of IL-1β occurs in two distinct steps. First, the signal 1 or "priming" signal leads to the synthesis of inactive precursor pro–IL-1β, and NOD-like receptor pyrin 3 (NLRP3), a protein important for IL-1β macrophages activation after stimulation by pathogen-associated molecular patterns (PAMPs), in particular LPS. The signal 2 or "activating" signal results in inflammasome formation, a sensor protein, which activates caspase-1 to cleave pro–IL-1β and allows secretion of mature IL-1β. Active IL-1β binds to IL-1 receptor type 1 (IL-1R1) and interplays with the IL-1 receptor accessory protein (IL-1RAP) to improve recruitment of myeloid differentiation factor 88 (MyD88) and IL-1 receptor-associated kinase 4 (IRAK4). This results in activation of different signaling pathways, such as NF-κB and mitogen-activated protein kinases (MAPK). IL-1β is capable of inducing various functions for macrophage activity by stimulating NF-κB activation, including inflammation, differentiation, apoptosis and cell proliferation [30,63]. In our study, we observed that GM-MDM treatment with Ss upregulated the production of IL-1β, but when combined with Met, the IL-1β production was downregulated. Herein, it would be advisable to check whether Ss would be able to act as an additional signal to stimulate the expression of pro–IL-1β and activation of NLRP3 inflammasome and caspase-1 in LPS-activated human macrophages, knowing that LPS is not enough to induce IL-1β release by human monocyte-derived macrophages in comparison to human circulating monocytes, which reflects their distinct roles in inflammatory conditions [64,65]. It would also be needed to evaluate mechanistically the role of Met alone and in combination with Ss on the regulation of NLRP3 inflammasome activation in human macrophages. So it has been recently reported in primary murine bone marrow-derived macrophages (BMDMs) that Met treatment inhibited in a dose-dependent manner LPS-induced pro-IL-1β mRNA and protein, and boosted induction of the anti-inflammatory cytokine IL-10 mRNA and protein in response to LPS, but had no effect on secretion of the mature form of IL-1β [61]. These results should pave the way for further research on the crosstalk between Met and Ss on the processed form of IL-1β. *In fine*, the comparison of the effects of Ss used alone against effects when combined with Met, would undoubtedly reinforce the important immunodominant effect of Met in human GM-MDMs under LPS-induced inflammatory conditions.

3.4. Effect of Met and Ss on the relative NADH dehydrogenase (complex I) levels in GM-MDM

As depicted in Fig. 5, treatment of GM-MDM with Met, Ss and Met + /Ss + markedly downregulated the relative levels of NADH dehydrogenase (complex I) (for all comparisons with untreated GM-MDM controls, $p < 0.001$). In addition, the relative levels of NADH dehydrogenase (complex I) were significantly increased in Met/Ss treated GM-MDMs when compared to Ss or Met-treated only GM-MDMs (respectively, $p < 0.01$ and $p < 0.01$). The comparison between means of different GM-MDM groups gave a significance level < 0.01 by

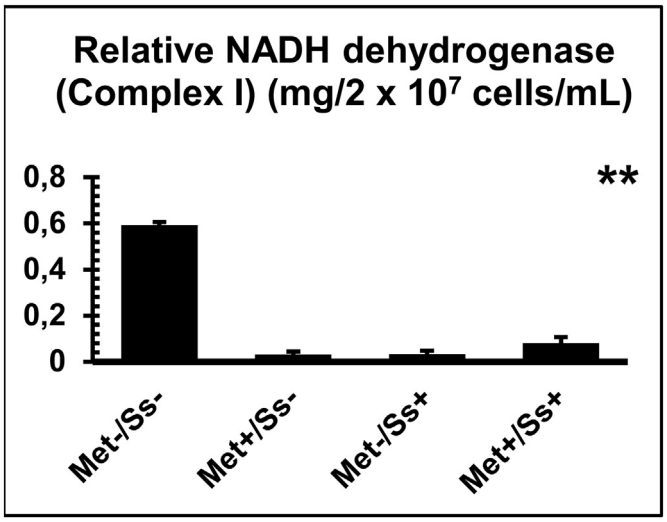

Fig. 5. Effect of Met and Ss on the relative NADH dehydrogenase (complex I) concentration in GM-MDM. The relative NADH dehydrogenase (complex I) concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using an appropriate commercial kit. $^{**}p < 0.01$ by Kruskal–Wallis test with pairwise comparisons using the Dunn-Bonferroni approach. The results are presented as mean values with standard error of mean (SEM) of four independent experiments carried out on cell samples isolated from three blood donors ($n = 12$ in each group). Met: metformin, Ss: sodium selenite, GM-MDM: granulocyte-macrophage colony-stimulating factor (GM-CSF) monocyte-derived macrophage, Met–/Ss–: untreated GM-MDM cell controls, Met + /Ss–: GM-MDM cells treated with metformin alone, Met–/Ss + : GM-MDM cells treated with sodium selenite alone, Met + /Ss + : GM-MDM cells treated with both metformin and sodium selenite.

Kruskal-Wallis test.

NADH dehydrogenase (complex I) (EC 1.6.5.3) is the first of four complexes of the mitochondrial membrane involved in the electron transport chain [61]. It allows electron entry in the oxidative phosphorylation system (OXPHOS) and NADH oxidation catalysis to generate a proton motive force that is used to synthesize ATP *via* the ATP synthase [61,66]. Our results have revealed that both Met and Ss induced a clear decrease in complex I concentrations. From the molecular point of view, Met can inhibit the complex I/NADH dehydrogenase of the mitochondrial respiratory chain as indirect activators of AMPK by inhibiting ATP synthesis, inducing accumulation of AMP and increasing the AMP-to-ATP ratio [18,67,68]. The activation of AMPK can result in increased production of mROS [20] and therefore confers a putative mutagenic risk [69]. In mitochondria, complex I have been reported to be the major ROS-generating site; nevertheless, sodium selenite or selenium supplementation may oppose ROS action and provide protective effects through increasing levels of glutathione peroxidase 1 (GPx1) and its free radical scavenging activity [70]. Conversely, another report indicated that complex I activity levels were slightly increased with selenium treatment to preserve mitochondrial functions [71]. Although the molecular interactions between complex I and Met have not yet been characterized, Met has been reported to be an effective inhibitor of mitochondrial respiratory complex I that remain the principal aspect of its therapeutic action [72], especially in the context of therapy against cancer development and tumorigenesis [73].

3.5. Effect of Met and Ss on $_{if}Ca^{2+}$ levels in GM-MDM

As shown in Fig. 6, the levels of $_{if}Ca^{2+}$ ions in Met and/or Ss treated GM-MDMs were decreased compared to untreated GM-MDMs (p-value was < 0.01 by Kruskal-Wallis test).

Calcium cations have been shown as key immune signals for macrophages in improving modulation of inflammation [34], as well as for proinflammatory cytokine release [74] and respiratory burst [30]. In addition, it has been demonstrated that an effective calcium flux influences the early immune response against bacteria, as it has been revealed in macrophages that Ca^{2+} restored bacterial recognition, phagocytosis and destruction of IgG-opsonized pathogens through Fcγ-receptor-mediated oxidative burst [74,75]. Conversely, reduction in intracellular calcium levels have been associated with promotion of immune evasion by decreasing macrophage ROS [74]. Recently, a study showed that Met inhibits islet function and decreased insulin secretion, mainly at the dose of 1 mM Met, which induced a marked shift in calcium and displayed clear enhancements in calcium modulation [76]. Additionally, intracellular rise in free calcium ions prompts increased oxidative stress and apoptosis [77]. It has previously been reported that intracellular Ca^{2+} concentration and release can be enhanced after selenium treatment in sarcoplasmic reticulum [78]. Nevertheless, to the best of our knowledge, there is no similar work allowing us to make comparisons.

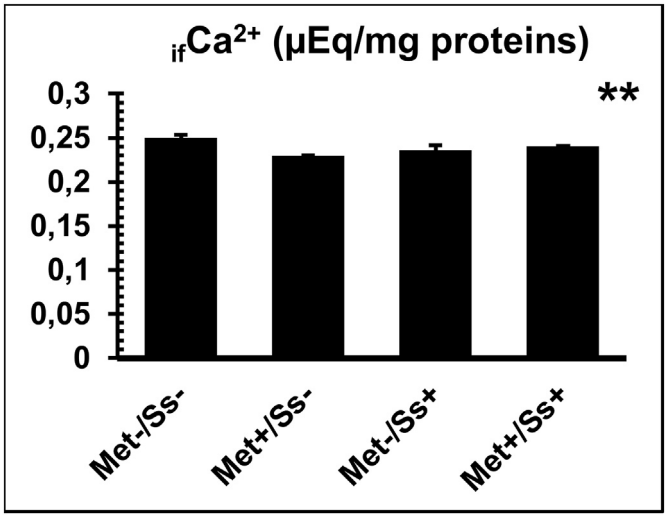

Fig. 6. Effect of Met and Ss on $_{if}Ca^{2+}$ levels in GM-MDM. Intracellular free calcium levels were measured spectrophotometrically using a commercial kit. $^{**}p < 0.01$ by Kruskal–Wallis test with pairwise comparisons using the Dunn-Bonferroni approach. The results are presented as mean values with standard error of mean (SEM) of five independent experiments carried out on cell samples isolated from three blood donors ($n = 15$ in each group). $_{if}Ca^{2+}$: intracellular free calcium ions, Met: metformin, Ss: sodium selenite, GM-MDM: granulocyte-macrophage colony-stimulating factor (GM-CSF) monocyte-derived macrophage, Met–/Ss–: untreated GM-MDM cell controls, Met + /Ss–: GM-MDM cells treated with metformin alone, Met–/Ss + : GM-MDM cells treated with sodium selenite alone, Met + /Ss + : GM-MDM cells treated with both metformin and sodium selenite.

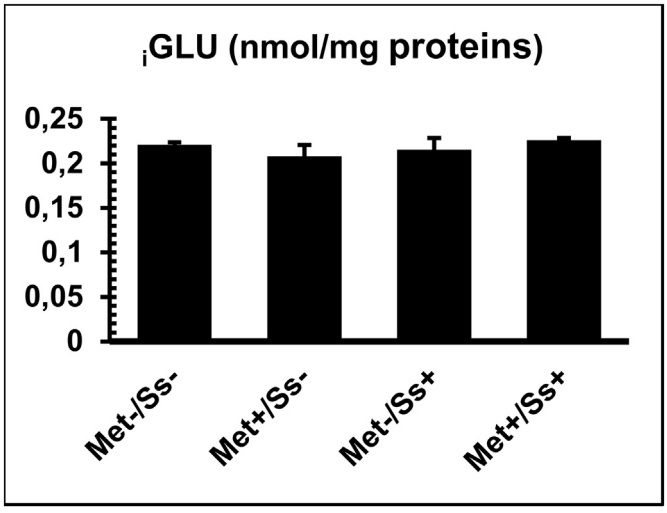

Fig. 7. Effect of Met and Ss on $_{i}GLU$ levels in GM-MDM. Intracellular glucose levels were spectrophotometrically measured based on the oxidation of glucose to gluconate by the glucose oxidase enzyme. No significant difference was shown by Kruskal–Wallis test with pairwise comparisons using the Dunn-Bonferroni approach. The results are presented as mean values with standard error of mean (SEM) of five independent experiments carried out on cell samples isolated from three blood donors ($n = 15$ in each group). $_{i}Glu$: intracellular glucose, Met: metformin, Ss: sodium selenite, GM-MDM: granulocyte-macrophage colony-stimulating factor (GM-CSF) monocyte-derived macrophage, Met–/Ss–: untreated GM-MDM cell controls, Met + /Ss–: GM-MDM cells treated with metformin alone, Met–/Ss + : GM-MDM cells treated with sodium selenite alone, Met + /Ss + : GM-MDM cells treated with both metformin and sodium selenite.

3.6. Effect of Met and Ss on $_{i}GLU$ levels in GM-MDM

As observed in Fig. 7, the levels of $_{i}Glu$ were significantly increased in GM-MDMs treated with Met + /Ss + in comparison to untreated controls ($p < 0.05$). Nevertheless, the remaining results have not reached the levels for statistical significance ($p > 0.05$). p-Value was > 0.1 using Kruskal-Wallis test.

Met can induce downregulation of gluconeogenic gene expression, and glucose-reduction in cells [18]. In addition, Met decreases glucose production by triggering important insulin-sensitizing mechanism [18,61]. On other hand, Met could inhibit hepatocellular glucose biosynthetic pathways by suppressing transcriptional steroid receptor coactivator 2 (SRC-2) [79]. Therefore, Met treatment has been shown to reduce growth of various cancers, including breast, colorectal, prostate, liver [18,80–82], lung and ovary cancer [83], especially through reducing insulin concentrations [20] since glucose is an essential nutrient that supports cancer cell growth and essential metabolism [84,85], or

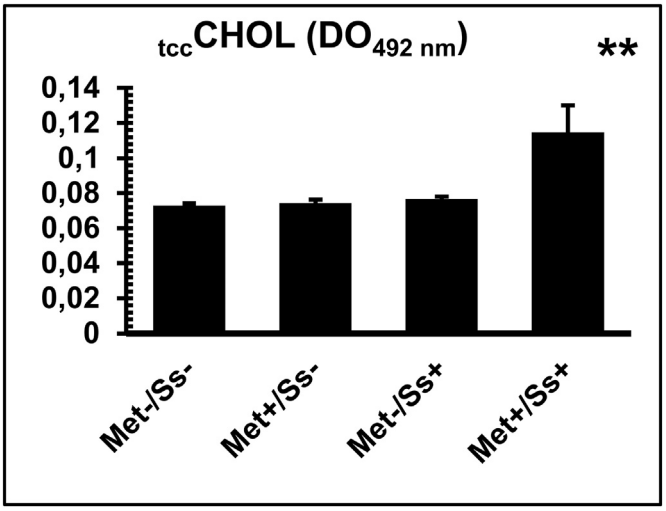

Fig. 8. Effect of Met and Ss on $_{tcc}$CHOL levels in GM-MDM. Total cholesterol content levels were measured with spectrophotometric method after conversion of cholesterol esters to free cholesterol by the enzyme cholesterol ester hydrolase (EC 3.1.1.1.13), using commercial reagents. $^{**}p < 0.01$ by Kruskal–Wallis test with pairwise comparisons using the Dunn-Bonferroni approach. The results are presented as mean values with standard error of mean (SEM) of five independent experiments carried out on cell samples isolated from three blood donors ($n = 15$ in each group). $_{tcc}$CHOL: total cholesterol content, Met: metformin, Ss: sodium selenite, GM-MDM: granulocyte-macrophage colony-stimulating factor (GM-CSF) monocyte-derived macrophage, Met–/Ss–: untreated GM-MDM cell controls, Met+/Ss–: GM-MDM cells treated with metformin alone, Met–/Ss+: GM-MDM cells treated with sodium selenite alone, Met+/Ss+: GM-MDM cells treated with both metformin and sodium selenite.

through suppression of mTORC1 *via* AMPK pathway activation [18,20]. Our results addressing the synergistic effect of Met and Ss treatment have shown a significant $_{i}$GLU increase, which could be related to increased glucose uptake. Under the same conditions, we also observed an increase in "M2" GM-MDM-associated arginase activity and in the ratio of arginase activity to iNOS activity, and a decrease in iNOS activity and IL-1β production, as mentioned above. Therefore, the increase in the levels of $_{i}$GLU might fuel alternative GM-MDM activation, following combined treatment with Met and Ss.

3.7. Effect of Met and Ss on $_{tcc}$CHOL levels in GM-MDM

We observed that $_{tcc}$CHOL levels were significantly increased in treated cells with Met+/Ss+ when comparing to untreated GM-MDM controls ($p < 0.01$) (Fig. 8). Similarly, levels of $_{tcc}$CHOL were more significantly increased in Met+/Ss+ treated GM-MDMs than in Met or Ss treated-GM-MDMs (for both comparisons, $p < 0.05$ by Mann-Whitney *U t*-test). When using the Kruskal-Wallis test, *p*-values were < 0.01.

It has already been established that cholesterol (CHOL) is involved in immune responses and cellular activation of various cell types, including macrophages [86]. This role is mainly effected through CHOL-enriched lipid rafts, dynamic microdomains located within the plasma membrane [87], that act as cell signaling platforms [30]. Additionally, CHOL has been considered as one of main activators of inflammasomes in macrophages leading, not only to IL-1β biosynthesis and M1 macrophage phenotype modulation, but also promoting endoplasmic reticulum (ER)-stress and subsequent apoptosis [88]. Nevertheless, the use of drugs acting on CHOL efflux could lead to pleiotropic and unexpected effects. Therefore, statins, anti-inflammatory drugs that induce CHOL lowering, have shown to directly active NLRP3 inflammasome and promote the production of IL-1β from macrophages, thus promoting metaflammation [89]. Conversely, we demonstrated in the present study that treatment with Met + Ss resulted simultaneously in decreased levels of IL-1β and in increased levels of both $_{tcc}$CHOL and the ratio of iNOS activity-to-arginase activity, which is usually related to enhancing activation of anti-inflammatory "M2" macrophage-associated arginase. These effects could occur through separate pathways, which can be beneficial for the regulation of IL-1β-induced inflammation, and at the same time for maintaining immune functions and activities of GM-MDMs-associated arginase, which could require a sufficient amount of cellular cholesterol. Moreover, although Met has been reported to be able to suppress CHOL biosynthesis [79], our results demonstrate that the combined effect of Met and Ss induced, conversely, an increase in $_{tcc}$CHOL levels. These data would prompt further investigations to verify whether the Met action has been circumvented or not by Ss treatment.

3.8. Effect of Met and Ss on the cell GM-MDM surface expression of CD80, CD86, CD14 and HLA-DR

In the present study, we used LPS as an exogenous antigen in conjunction with Met combined or not with Ss and then evaluated the expression levels of co-stimulatory molecules and HLA-DR on the cell surface. GM-MDM's expression levels of co-stimulatory molecules CD80, CD86, cell activation and molecule presentation including CD14 and human leukocyte antigen–DR (HLA-DR), were evaluated by FACS in LPS-activated GM-CSF monocyte-derived macrophages, treated with Met and/or Ss together with untreated isotypes control cells. "Isotype controls" are untreated cells with isotypes antibodies. As shown Fig. 9, the histograms and dot plots reveal that Met induced a substantial increase in CD80 expression and a slight increase in CD14 expression and CD14 and CD86 co-expression. Conversely, Met treatment induced a decrease in HLA-DR expression, and CD80 and HLA-DR co-expression. Additionally, Ss treatment induced a slight increase in CD14, CD80, and HLA-DR expression, and CD80 and HLA-DR co-expression. In contrast, treatment with Ss induced a slight decrease in CD86 expression and CD14 and CD86 co-expression. Moreover, the Met/Ss combination accentuated the effects of Ss treatment, resulting in a strong increase of CD14 and HLA-DR expression and CD80 and HLA-DR co-expression as well as a sharp decrease in CD86 expression and CD86 and CD14 co-expression that was accompanied by upregulation of CD80 expression.

Of note, one of the important roles played by macrophages is the activation of naive T-cells and the generation of primary T-cell responses [90]. Subsequently, activated macrophages express high levels of co-stimulatory and antigen-presenting molecules like, CD80/CD86 and HLA-DR, respectively [2,4,91]. CD80 and CD86 are two important co-stimulatory molecules part of the B7 family and members of the immunoglobulin supergene family (IgSF) [92]. The expression of CD80 on APCs can promote T helper type 1 (Th1) cell differentiation, whereas the expression of CD86 has been shown to be involved in the differentiation of Th2 cell phenotype [93]. It has been reported that dysregulation of CD80/86 expression may influence the development of immune responses, including production of cytokines in response to stimulation with toll-like receptor 4 (TLR-4) ligand, *i.e.* LPS [68]. On the other hand, CD14 is a well-known monocyte/macrophage surface marker [94] and LPS binding protein that can induce inflammatory responses [95]. A recent study performed on mice showed that Met inhibited the expression of exogenous antigen-presenting capability of peritoneal-macrophages and suppresses both MHC molecules and CD80 and CD86 [90]. However, other studies suggested that selenium is a dose-dependent inhibitor of HLA-DR expression on thyrocytes induced by interferon-γ, knowing that, similarly to macrophages, thyrocytes are able to express a HLA class II antigen, which suggested the protective effect of selenium against the development of autoimmune disorders [96]. Based on our results, Met treatment appears to have substantial effects on the cell surface expression of both HLA-DR and CD80, but lesser effects on CD14 expression and CD14 and CD86 co-expression on LPS-stimulated human GM-MDM. A recent study performed on

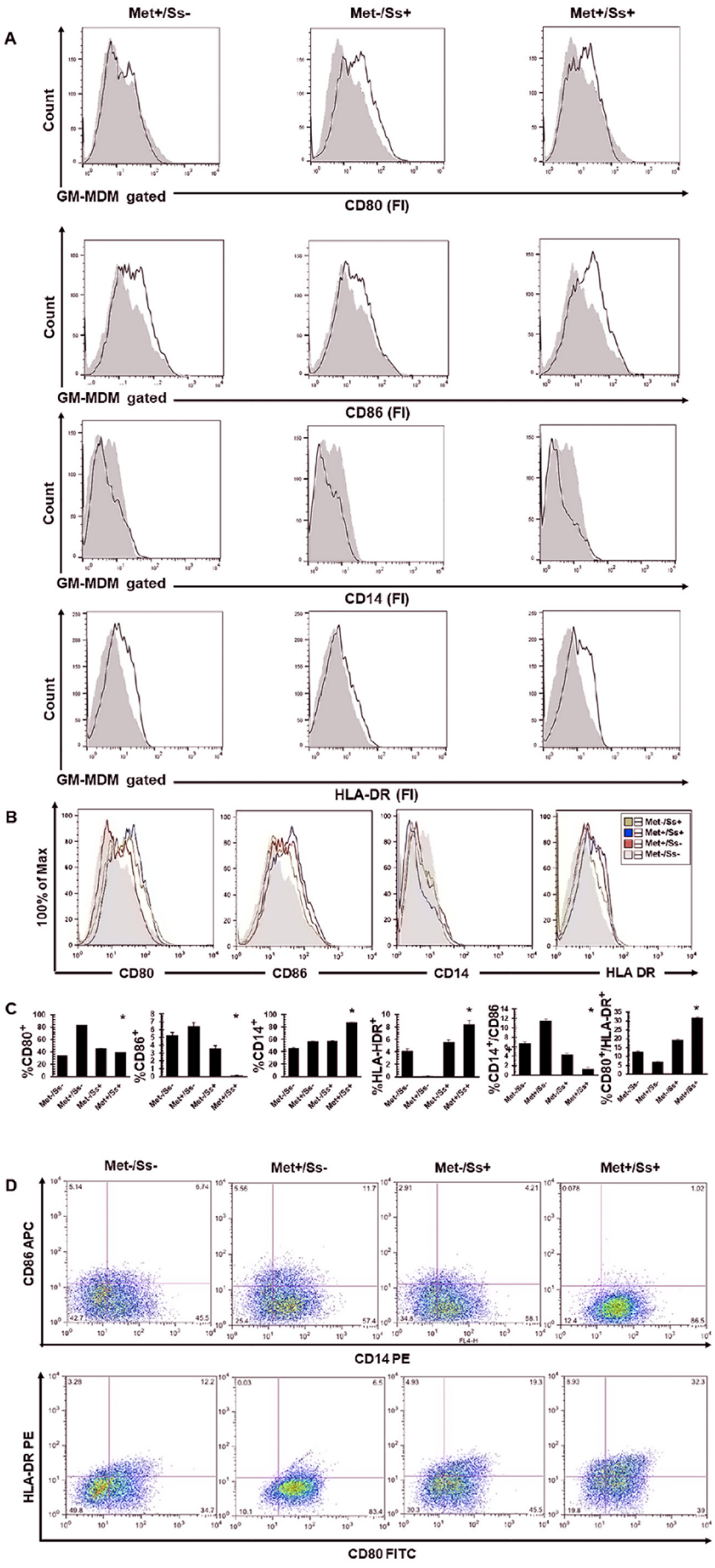

Fig. 9. Effect of Met and Se on the cell GM-MDM surface expression of B7-1 (CD80), B7-2 (CD86), CD 14 and HLA-DR. GM-MDMs were first stimulated with LPS for 24 h and were left untreated or treated for 24 h with Met and/or Ss. Surface expression of CD80, CD86, CD14 and HLA-DR (major histocompatibility complex class II cell surface receptor encoded by the human leukocyte antigen) were assessed by flow cytometry. (A) Histograms are illustrative of Met or Ss treated cells (black lines) compared with untreated isotype controls cells (filled gray areas). Experiments were performed in duplicate on cell samples isolated from three blood donors and are representative of three independent experiments with similar results ($n = 9$ in each group). (B) Histogram overlays are displayed as % Max, scaling each curve to mode 100%; the red line is the Met + /Ss– treated-cells, while the blue line are Met + /Ss + , the green line are Met–/Ss + and the filled gray areas are the Met–/Se– untreated controls. All groups were set on the scale to 100 for revealing changes in the cell distribution. (C) Percentage of CD80, CD86, CD14 and HLA DR cell surface activation marker expression on macrophages were analyzed by flow cytometry. Mean results with SEM of cells expressing compared with untreated isotype controls cells of three different donors assayed in duplicate are shown. *p-value < 0.05 by Kruskal–Wallis test with pairwise comparisons using the Dunn-Bonferroni approach. (D) Flow cytometry raw scatter plots are showing the percentage of CD80, CD86, CD14 and HLA-DR expression for the gated GM-MDM populations after Met and/or Se treatments. The results were analyzed using Flowjo software 7 (Tree Star Inc., Ashland OR). Met: metformin, Ss: sodium selenite, GM-MDM: granulocyte-macrophage colony-stimulating factor (GM-CSF) monocyte-derived macrophage, Met–/Ss–: untreated isotype controls cells, Met + /Ss–: GM-MDM cells treated with metformin alone, Met–/Ss + : GM-MDM cells treated with sodium selenite alone, Met + /Ss + : GM-MDM cells treated with both metformin and sodium selenite. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dendritic cells and APCs reported that Met simultaneously decreased the expression of total MHC molecules, by decreasing their MHC class II-restricted exogenous antigen-presenting capability, and suppressed expression of CD80 [90]. Both these observations are in accordance with our findings regarding the co-expression levels of CD80 and HLA-DR. Our results also showed that Ss treatment may influence, and even dominate or counter the effects exerted by Met alone on the expression of CD14, CD80, CD86 and HLA-DR. Interestingly, the use of combined therapy of two or more molecules has shown some advantages over monotherapy, as demonstrated recently by a combination of Met and Lovastatin in experimental autoimmune encephalomyelitis [97], or Met and sodium selenite, used as an adjunct, in the treatment of type 2 diabetes [98]. Moreover, combination therapy of selenium with glucan has been demonstrated to enhance immunomodulatory effects against cancer growth by stimulating immunity, including phagocytosis [99], which may corroborate our results regarding the upregulation of the expression of CD14, which can potentially be required for the induction of phagocytosis [100] as an accessory receptor.

4. Conclusions and future prospects

Met and selenium are prominently two distinct bioactive molecules regarding their therapeutic applications and mechanisms of action. Yet, they have common properties with potential to modulate the immune response and immunometabolism and to induce a functional phenotype switch of human GM-MDMs. This study supports the hypothesis that both Met and selenium, and especially in combination can have a remarkable role to play in the modulation of the overall functional activities of human classically activated/LPS-stimulated GM-MDMs. In summary, compared to Met and Ss used separately, the combined effect of Met and Ss can induce “M2” GM-MDM-associated arginase. Additionally, Ss appears to influence or even dominate markedly the effects of Met on the functional activities of human GM-MDM, as well as the surface expression of co-stimulatory and cell activation and presentation molecules. Finally, since the Met + Ss combination has been associated with increased levels of $_{tcc}$CHOL, it will be of great interest to determine the direct effect of such a combination on the expression of the gene that modulates the expression of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, which is involved in the CHOL biosynthesis pathway.

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Conflict of interest

The authors declare no conflict of interest.

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