


# Aspirin 15cH has Different Effects on Morphology and Function of Lipopolysaccharide-Challenged RAW 264.7 Macrophages *In Vitro* Compared to a Pharmacological Dose of Aspirin

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## Abstract

**Introduction** Aspirin is one of the most commonly used drugs worldwide. It is known to present antipyretic, anti-inflammatory and anti-thrombotic actions, making it extremely useful in a wide range of clinical contexts. Interestingly, homeopathically prepared *Aspirin* 15cH has been found to have a pro-thrombotic effect in rats, raising the hypothesis that *Aspirin* 15cH could also modulate the activity of inflammatory cells in different pathological processes.

**Objective** Our objective was to assess what effect *Aspirin* 15cH has on RAW 264.7 macrophages *in vitro*.

**Methods** The effects of *Aspirin* 15cH on biochemical and morphological activities of lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages were evaluated. These effects were compared with unchallenged macrophages (negative control), untreated LPS-stimulated macrophages, macrophages treated with succussed water (vehicle control), or aspirin 200 µg/mL (pharmacological inhibitor of LPS activity). Cell morphology (adhered cell area and cytoskeleton arrangements), cell viability, toll-like receptor-4 (TLR-4) expression, and the production of nitric oxide, cytokines and intracellular reactive oxygen species were assessed.

**Results** *Aspirin* 15cH reduced the number of cells expressing TLR-4 on the surface ( $p = 0.03$ ) and induced a “columnar” morphology of macrophage pseudopods, indicating changes in cytoskeleton arrangement. When cells were treated with both *Aspirin* 15cH and LPS, cell morphology became heterogeneous, suggesting that sub-populations of cells had differing sensitivities to LPS or *Aspirin* 15cH. Exposure of the cells to

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LPS alone, succussed water or aspirin 200 µg/mL produced effects consistent with the literature.

**Conclusion** *Aspirin* 15cH, aspirin 200 µg/mL, LPS and succussed water appear to act as independent stimuli able to induce different patterns of macrophage response. *Aspirin* 15cH induced changes suggestive of M2 polarization of the macrophages (i.e., toward a wound healing or tissue repair, rather than inflammatory, phenotype). These preliminary findings need to be confirmed in further specific studies.

## Introduction

Aspirin has been one of the most-used drugs worldwide for many years due to its broad sphere of action. Aspirin is known to act as an antipyretic, anti-inflammatory, and/or anti-thrombotic agent.<sup>1–6</sup> The main mechanism of action of aspirin is well established: that is, aspirin acts through inhibition of the cyclooxygenase enzymes COX-1 and COX-2, responsible for converting arachidonic acid to prostaglandins and thromboxane, which in turn cause platelet aggregation, inflammation, fever and pain. COX-1 is constitutively expressed, whereas COX-2 can be induced by inflammatory stimuli. For this reason, aspirin can induce multiple and simultaneous changes in inflammatory cells, vascular endothelium and platelets, which impact the production of cytokines, thrombin and platelet-activating factor, and the expression of endothelial adhesion molecules and macrophage toll-like receptors (TLR-4).<sup>7–12</sup> Moreover, when aspirin is used as an anti-platelet therapy, it can present some paradoxical effects in patients: the so-called phenomenon of aspirin resistance<sup>3,13–15</sup>. Abrupt discontinuation of treatment can also culminate in pro-thrombotic events associated with a withdrawal syndrome,<sup>16,17</sup> thus highlighting the far-reaching effects of aspirin as a pharmacological agent.

The need to understand this paradoxical activity of aspirin motivated Doutremepuich and collaborators to carry out a series of studies from 1987 to 2019 on dose-dependent aspirin effects, which included highly diluted and potentized preparations.<sup>18–27</sup> Additionally, Aguejoui et al.<sup>22</sup> observed that *Aspirin* 15cH (a succussed dilution of  $1.25 \times 10^{-29}$  M) inhibited the anti-thrombotic effect of indomethacin—a non-specific inhibitor of cyclooxygenases—only one hour after its administration. Eizayaga et al. later suggested a specific participation of COX-2 in the *Aspirin* 15cH effects.<sup>24</sup> These findings raised the hypothesis that *Aspirin* 15cH could potentially also modulate the activity of inflammatory cells, directly in thrombosis or in other pathological processes. No report was found in the literature regarding this issue.

Thus, the present study aimed to evaluate whether *Aspirin* 15cH can regulate RAW 264.7 macrophage function *in vitro*, with or without lipopolysaccharide (LPS) challenge. Therefore, biochemical and morphological parameters were analyzed, and different controls were used as biological references to identify the specificity of any effects seen.

## Materials and Methods

### Solution Preparation

*Aspirin* 14cH (as stock dilution or from a matrix of lower-potency dilutions) was prepared in a pharmacy registered by the Brazilian Sanitary Surveillance Agency. The manufacturer followed the Brazilian Homeopathic Pharmacopeia. The stock dilution was prepared by adding 1 g of pure powdered aspirin (Bayer, Whippany, United States) to 99 mL of 70% hydroalcoholic solution prepared with distilled and sterilized water, using a new and autoclaved 30 mL amber type 2 glass flask, as conventionally used for medicinal preparations. The solution underwent 100 vigorous vertical shakings (succussion) in an automatic device (Denise-Autic, São Paulo, Brazil). Next, 1 mL of this solution was mixed with another 99 mL of 70% hydroalcoholic solution using the same flask type and submitted to a new shaking cycle. This last step was repeated 12 more times until reaching the 14th centesimal dilution, which was then stored as a stock solution. On the eve of each new experiment, the last working dilution was made in sterile purified water obtained from SmartPak Direct Q3 and Biopak filters (Merck-Millipore, Darmstadt, Germany), autoclaved and submitted to 100 automatic succussions to obtain sterile *Aspirin* 15cH. The final theoretical concentration of acetylsalicylic acid was thus  $1.25 \times 10^{-29}$  M.

An identical flask containing sterile water from the same batch was submitted to 100 succussions using the same device, to be used as vehicle control (named succussed water) since it can mimic the last step used in *Aspirin* 15cH preparation.

A pharmacological dose of aspirin was prepared in sterile RPMI 1640 medium to be added into the culture to reach the final concentration of 200 µg/mL.<sup>28</sup> It was used as a pharmacological inhibitor of LPS to check the internal validity of the experimental model regarding the LPS challenge.

Aliquots of LPS (Sigma-Aldrich, Saint Louis, United States) were also prepared in RPMI 1640 medium, resulting in a 1 µg/mL stock solution. The final concentration of LPS used in all experiments was 0.1 µg/mL.

All glass vials were sterilized, and all drugs and controls were filtered in microfilters for 0.22 µm mesh syringes (Kasvi, São José dos Pinhais, Brazil) before being introduced into the cell culture to ensure their sterility as far as possible.

### Cell Culture

RAW 264.7 macrophages (Cell bank, Rio de Janeiro, Brazil) are an immortalized cell line obtained from a hybridoma

originally produced from peritoneal macrophages of Balb/c mice. A p22 passage<sup>29</sup> was kept frozen at  $-80^{\circ}\text{C}$  up to the beginning of the experiments. After thawing and washing, cells were expanded in standard bottles (Kasvi, São José dos Pinhais, Brazil) with ventilated lids, containing RPMI 1640 (Gibco, Waltham, United States) supplemented with 10% fetal bovine serum (FBS; Cutilab, Campinas, Brazil)—i.e., “R10 medium”—at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . No other additives were inserted into the culture.

After attaining 90% confluence, cells were washed with an RPMI 1640 medium, detached from the bottom of the bottle with a cell scraper (Kasvi, São José dos Pinhais, Brazil), washed, and re-suspended. Cell viability was checked with 0.4% trypan blue using an automatic cell counter (Countess, Thermo Fisher Scientific Company, Waltham, United States) using a specific chamber supplied by the manufacturer. Suspensions of cells at a known concentration were then used according to the required assay (microscopy, supernatant analysis, or MTT assay), or cells were maintained in the bottles and treated according to the experimental group for flow cytometry.

### Experimental Design

Cells and treatments were distributed in seven experimental groups, as described in ►Table 1.

### Cell Morphology

Cell morphological analysis was performed using two different assays: quantitative analysis of Giemsa-stained slides via light microscopy and qualitative assessment of fluorescent-labeled cells by confocal microscopy.

Cells suspensions (as detailed in section 2.2) were transferred to a 24-well plate at  $2 \times 10^5$  cells/mL per well with a sterile rounded glass coverslip on the bottom to allow cell adhesion. The R10 medium was made up to a total volume of 300  $\mu\text{L}$ , and cells were incubated at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  for 2 hours for complete adhesion. Treatments were then added according to each experimental group (►Table 1) and incubated for 24 hours.

For light microscopy, the cells adhered to the coverslips were fixed with absolute methanol (Synth, Diadema, Brazil) for 15 minutes and stained with a Giemsa ready-to-use stain (Laborclin, São José deo Rio Preto, Brazil) diluted as one drop for each 1 mL of distilled water, according to the manufac-

turer's recommendation. Then, slides were washed and mounted on coverslips with Entellan resin (Merck-Millipore, Darmstadt, Germany).

Twenty fields per coverslip were then assessed using sequential optical fields, and the average adhered cell area (pixels per cell) was quantified using the image analysis software Metamorph off-line version (Molecular Devices, San José, United States). Adhered cell area is a measure of the macrophage activation process called “cell spreading.”<sup>30</sup> The samples were analyzed in sextuplicate in two series of independent experiments.

A second series of experiments on cell morphology was performed to further characterize the process of “cell spreading” via 3D visualization of the cytoskeleton arrangements by F-actin staining. Thus, after 24 hours of treatment and incubation in 24-well plates, as described above, cells were fixed with a 3.7% formaldehyde-PBS (phosphate buffer saline) solution (Sigma-Aldrich, Saint Louis, United States) for 10 minutes, washed in PBS, permeabilized with a 0.1% Triton-X/PBS solution (Sigma-Aldrich, Saint Louis, United States), and incubated for another 10 minutes. Coverslips were washed with PBS and stained with ready-to-use 2.5% phalloidin conjugated with Alexa Fluor 488 (Life Technologies/Molecular Probes Inc., Eugene, OR, United States) in PBS with 10  $\mu\text{L}$  DAPI-Fluorishield 4',6'-diamino-2-phenyl-indol (Sigma-Aldrich, Saint Louis, United States). The culture plates were maintained in the dark for 24 hours at room temperature to allow the staining and assessed by laser scanning confocal microscopy (Carl Zeiss, Jena, Germany). Confocal images were acquired on a Zeiss LSM-780 NLO (CEFAP, ICB—USP, FAPESP 2009/53994-8) using a  $60 \times 1.4$  NA oil immersion lens (Objective Plan-ApoChromat 63  $\times$  /1.40 Oil Infinity/0.17). Z-stack images of representative fields of each sample were taken with optical slices spaced at 5  $\mu\text{m}$  intervals from the bottom to the top of the cells. The images were then processed using the Imaris software (Bitplane, Zurich, Switzerland), in which the functions *surpass scene/volume/snapshots/animations* were used to convert serial images to mp4 files.

### Cytotoxicity

Cytotoxicity of all treatments was assessed using the MTT (Thiazolyl Blue Tetrazolium Bromide) method (Sigma-Aldrich,

**Table 1** The experimental design by groups and treatments

Group	R10 Medium	Lipopolysaccharide	Treatment
Unchallenged	+	—	—
Vehicle control	+	—	Succussed water
LPS challenge (1 $\mu\text{g}/\text{mL}$ )	+	+	—
Unchallenged cells with potentized aspirin	+	—	<i>Aspirin</i> 15cH
Challenged cells with potentized aspirin	+	+	<i>Aspirin</i> 15cH
Challenged cells with vehicle control	+	+	Succussed water
LPS inhibition	+	+	Aspirin (200 $\mu\text{g}/\text{mL}$ )

Abbreviation: LPS, lipopolysaccharide.

Saint Louis, United States). This method tests the ability of the mitochondrial enzyme succinate dehydrogenase to reduce tetrazolium salt into formazan, which can be quantified by colorimetry. In 96-well culture plates, each experimental group consisted of four wells in which  $2 \times 10^5$  cells were placed with the respective treatments added to the medium just before seeding. After 24 hours of incubation at 37°C, 5% CO<sub>2</sub> to allow cell adhesion and stabilization, the supernatants were removed, the cells were washed in PBS, and 100 µL of 0.5 mg/mL tetrazolium solution (MTT reagent) was added to each well. The plate was incubated for another 4 hours in the same conditions, and 100 µL of dimethyl sulfoxide was added for crystal solubilization. The plate was gently shaken for 15 minutes before reading in an enzyme-linked immunosorbent assay (ELISA) reader (Thermo Fisher Scientific Company, Waltham, United States) at 570 nm. The experiment was performed in triplicate.

### TLR-4 Expression

The measurement of TLR-4 expression on the cell surface was made by flow cytometry. Three experimental replicates were done (each with three repetitions), in which  $2 \times 10^5$  cells per bottle were distributed and maintained at 37°C, 5% CO<sub>2</sub> for 24 hours for cell adhesion and expansion. Cells then received the appropriate treatment, with or without LPS, and were incubated for 24 hours. LPS was inserted into the R10 medium (1 µg/mL), and treatments were simultaneously inserted in a 1:30 ratio.

The supernatants were removed and discarded, and the adhered cells were washed with RPMI 1640 and lifted in R10 medium with a cell scraper. Then, they were transferred to a Falcon tube and centrifuged for 7 minutes at 0.3 rcf (relative centrifugal force). All steps of cell suspension manipulation were done on ice to avoid cell adhesion to the tube's wall. The supernatants were discarded, and the pellets were re-suspended in 1% Bovine Serum Albumin (BSA) (Sigma-Merck, Saint Louis, United States) in PBS in a volume necessary to reach  $1.5 \times 10^6$  cells/mL. Next, 300 µL of cell suspension was added to microtubes containing 3 µL of mouse Fc-block (BD, New Jersey, United States) and incubated at 4°C for 20 minutes.

Subsequently, each tube received an additional 200 µL of 1% BSA in PBS. Cells were gently mixed and equally distributed into three other microtubes, 167 µL each, intended for the sample triplicates. The microtubes were centrifuged again for 7 minutes at 0.3 rcf, the supernatants were discarded, and the cells were re-suspended in 30 µL of a rat monoclonal antibody solution (1 µg/mL). The monoclonal antibody was raised against the TLR-4/MD-2 complex of mouse origin and conjugated with phycoerythrin (sc-13591 PE; Santa Cruz Biotech, Santa Cruz, CA, United States). Samples were incubated at 4°C for 20 minutes, protected from light.

After the incubation, the microtubes were centrifuged, washed with a sterile PBS solution, and 200 µL of PBS was added to re-suspend the cell pellet. Acquisition and analysis were performed on an ACCURI C6 flow cytometer (BD biosciences, New Jersey, United States): 20,000 events

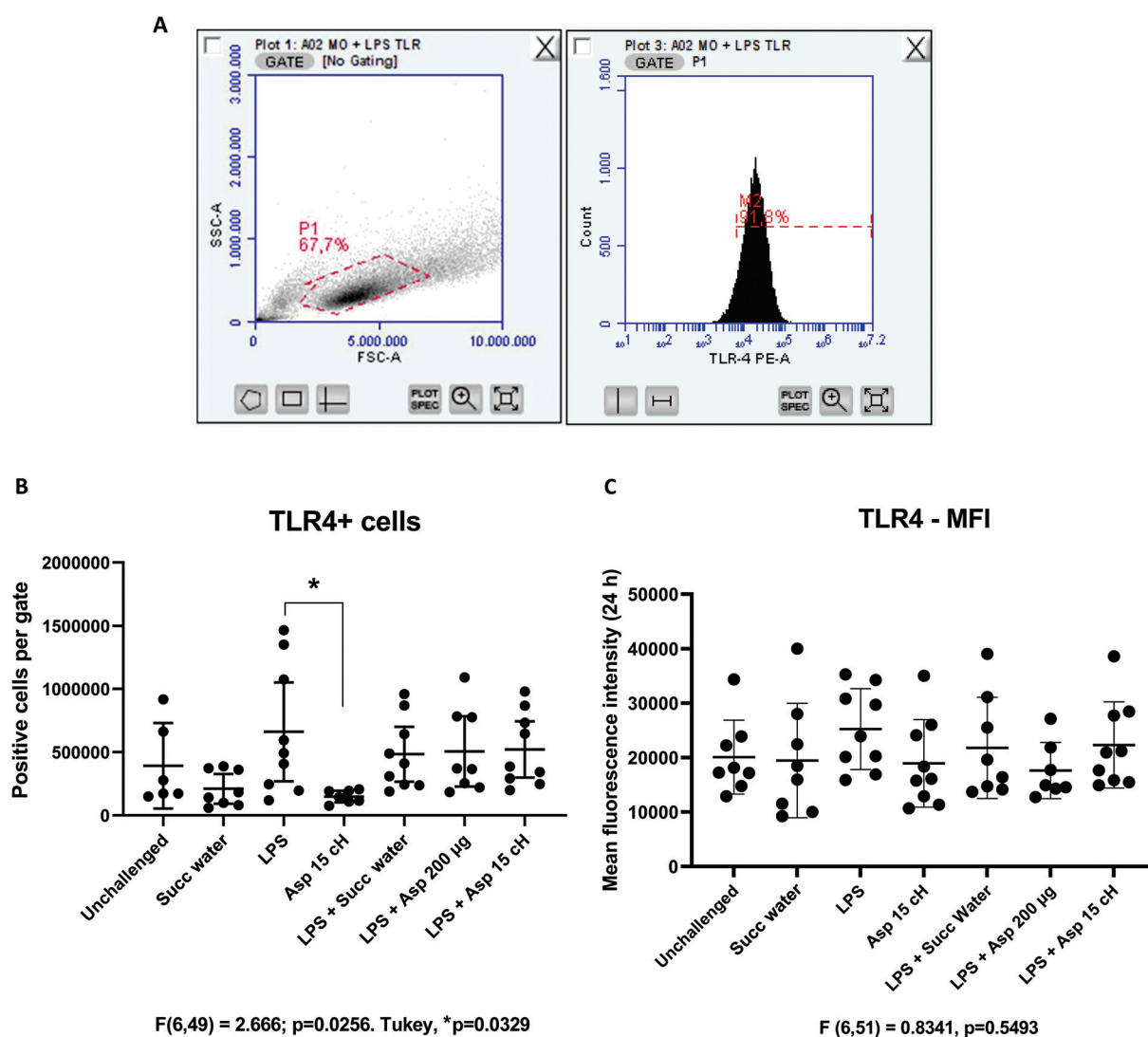
were collected per sample and used to ascertain the number of positive cells in a gated area. This value was then used to calculate the absolute number of positive-labeled cells in 200 µL (total sample volume). In addition to the number of TLR-4-positive cells per gate (see ►Fig. 1A), the arithmetic mean fluorescence intensity (MFI) per cell of TLR-4 expression was also measured. The raw fluorescence data and cell number calculations were automatically created in a sheet generated by BD ACCURI C6 software (BD biosciences, New Jersey, United States).

### Indirect Nitric Oxide Measurement

The supernatants from cells cultured in 24-well plates (see Section 2.4) were harvested and stored at –80°C. These supernatants were then used to assess nitrite levels (NO<sub>2</sub><sup>–</sup>), a metabolite of NO produced by macrophages in the culture, which can be used as an indirect measurement of NO production. Each sample was evaluated in two series of sextuplicates, using 96-well microplates. Thus, 100 µL of the supernatant and 100 µL of Griess' reagent (0.1% N-1-naphthylethylenediamine dihydrochloride [NEED; Sigma-Merck, Saint Louis, United States], 1% sulfanilamide, and 2.5% phosphoric acid) were mixed in each well. The plates were left for 10 minutes at room temperature and read in an ELISA reader (Thermo Fisher Scientific Company, Waltham, United States) at 540 nm. The results were expressed as µM of nitrite for  $5 \times 10^5$  cells. A standard curve was made from the source solution, 100 µM sodium nitrite (Sigma-Merck, Saint Louis, United States). The data points were obtained from decreasing concentrations reaching 0 µM and plotted on a scatter plot to generate a linear equation. Results were expressed as optical density and compared with the standard nitrite curve to calculate the actual concentrations in the sample.

### Cytokine Concentration in Supernatants

The supernatant from cells cultured in 24-well plates (see section 2.4) was harvested and stored at –80°C. These supernatants were then used to assess cytokine concentrations, as follows. The supernatants were thawed and microcentrifuged at 1,500 rpm (Hettich 32A, Tuttlingen, Germany) for 5 minutes to remove cell debris. The samples were analyzed in triplicate. The commercially available Cytometric Bead Array (CBA) mouse Inflammation® kit (BD Biosciences, New Jersey, United States) was used to quantify the production of a panel of six cytokines: interleukin-6 (IL-6), IL-10, monocyte chemoattractant protein-1 (MCP-1), gamma-interferon (γ-IFN), tumor necrosis factor-α (TNF-α), and IL-12 p70 (IL-12 p70). Briefly, the CBA provides tiny beads bound with antibodies to specific cytokines; these cytokines are thus captured and available for secondary fluorescent labeling (by the sandwich method) and detection by flow cytometry. The intensity of PE fluorescence of each sandwich complex reveals the concentration of cytokine. After acquiring samples on a flow cytometer, the FCAP Array software generates results in pg/mL. The samples were analyzed according to the instructions provided by the manufacturer. Samples below the detection limit were not recorded.



**Fig. 1** (A) Scattering plot and histogram obtained from BD Accuri C6 flow cytometer showing the gate defined for viable LPS challenged macrophages. P1 represents the macrophage gate, from which the sub-population M2 was identified. M2 represents the percentage of positive cells per gate. (B) TLR4-positive RAW 264.7 macrophages under different challenges and treatments, evaluated by flow cytometry. One way ANOVA,  $F_{(6,49)} = 2.666$ ,  $p = 0.0256$ . Tukey test,  $*p = 0.0329$  in relation to LPS. Seven outliers were withdrawn, being two from Asp 15cH, three from unchallenged, one from succussed water, and one from LPS + Asp 200 µg/mL. (C) Arithmetic mean fluorescence intensity (MFI) of RAW 264.7 macrophages, under different exposures, obtained from BD Accuri C6 flow cytometer and processed using Flow Jo software. One way ANOVA,  $F_{(6,51)} = 0.8341$ ,  $p = 0.5493$ . Five outliers were withdrawn. Data are represented by mean, standard deviation and single data points, considering the absolute cell number in samples of 200 µL.

### Reactive Oxygen Species Production

A 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCF-DA) (Sigma-Merck, Saint Louis, USA) probe was used to quantify reactive oxygen species (ROS) production using flow cytometry. It is a fluorometric dye capable of detecting the presence of intracellular ROS as an indirect way to evaluate cell oxidative activity. Cell suspensions were prepared in the same way as described in item 2.6. Two experimental series were performed, each one with three replicates.

From a volume of 300 µL containing  $1.5 \times 10^6$  cells/mL suspended in 1% BSA (Sigma-Merck, Saint Louis, United States) in PBS, aliquots were divided into three microtubes (which received a volume of 100 µL) containing  $5 \times 10^5$  cells each. The microtubes were centrifuged for 7 minutes at

0.3 rcf, and cells were re-suspended in a 1 µM solution of the probe (5 µL probe at 100 µM + 495 µL of PBS) and incubated at 37°C for 30 minutes. Subsequently, the cells were centrifuged and washed in PBS twice. Finally, the cells were re-suspended in 200 µL of PBS and taken to the flow cytometer for quantification of positive cells and fluorescence intensity (using the FITC-green channel) as detailed in section 2.6.

### Statistical Analysis

All quantitative results are presented as mean ± standard deviation. The GraphPad Prism 9.5.0 program performed the statistical analysis and was used to generate graphs. The Shapiro-Wilk test was used to evaluate the Normality of the sample values. Outliers were withdrawn after QQ-plot inspection and checked according to Tukey's criteria using



the GraphPad Prism 9.5.0 graphic tool. One-way analysis of variance (ANOVA) and Tukey's post-test were used to verify statistical significance, being set at  $\alpha = 0.05$ . F-value followed by the universe of independent determinations, expressed in degrees of freedom (between groups; within groups), was also used to describe the statistical data.

### Ethics Statement

Approval by an ethical committee was not required to enable the conduct of this study: RAW 264.7 macrophages are a commercially available murine cell line, and no animals or primary cells were used.

## Results

### Light Microscopy Assessment of Cell Morphology

The quantitative analysis of Giemsa-stained macrophage adhered cell area measured in pixels reveals different response patterns according to treatment and challenge (► Fig. 2).

Challenging macrophages with LPS (a potent polarizer toward the M1 pro-inflammatory phenotypic profile) produced an increase in mean adhered cell area ( $p \leq 0.03$ ), as expected. The same cell area increase was observed with Aspirin 15cH treatment alone, but with a different morphological pattern, as described in Section 3.2 below.

The effect of succussed water was intermediary: no statistically significant difference was seen compared with unchallenged cells or LPS and Aspirin 15cH-treated cells, but, paradoxically, when LPS challenge was combined with succussed water, a significant decrease in cell area was seen. This was comparable to the effects seen after the treatment with aspirin 200  $\mu\text{g/mL}$ , which was statistically lower than the

baseline (unchallenged cells) ( $p \leq 0.03$ ). Taken together, this is suggestive of non-specific effects of succussed water.

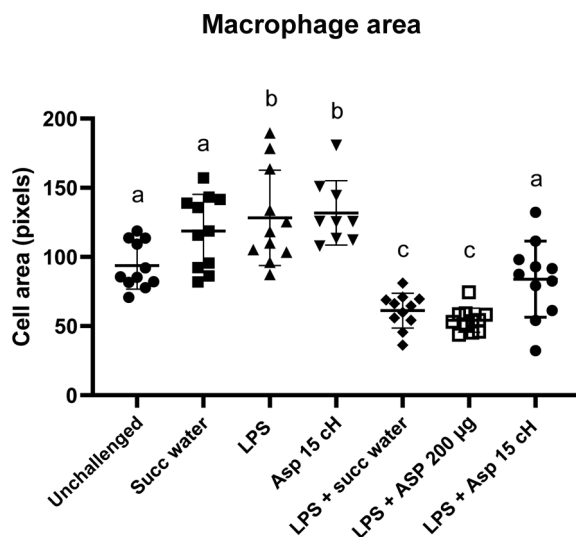
The treatment with combined LPS and Aspirin 15cH produced a less intense inhibitory effect, comparable to the baseline but statistically different from cells treated with LPS or Aspirin 15cH only ( $p \leq 0.03$ ).

### Confocal Microscopy Assessment of Cell Morphology

Confocal microscopy was used to allow a three-dimensional evaluation of cytoskeletal arrangements via F-actin distribution in the membrane-cytoplasm interface, to further explore the morphological changes seen by light microscopy in LPS-challenged and treated cells.

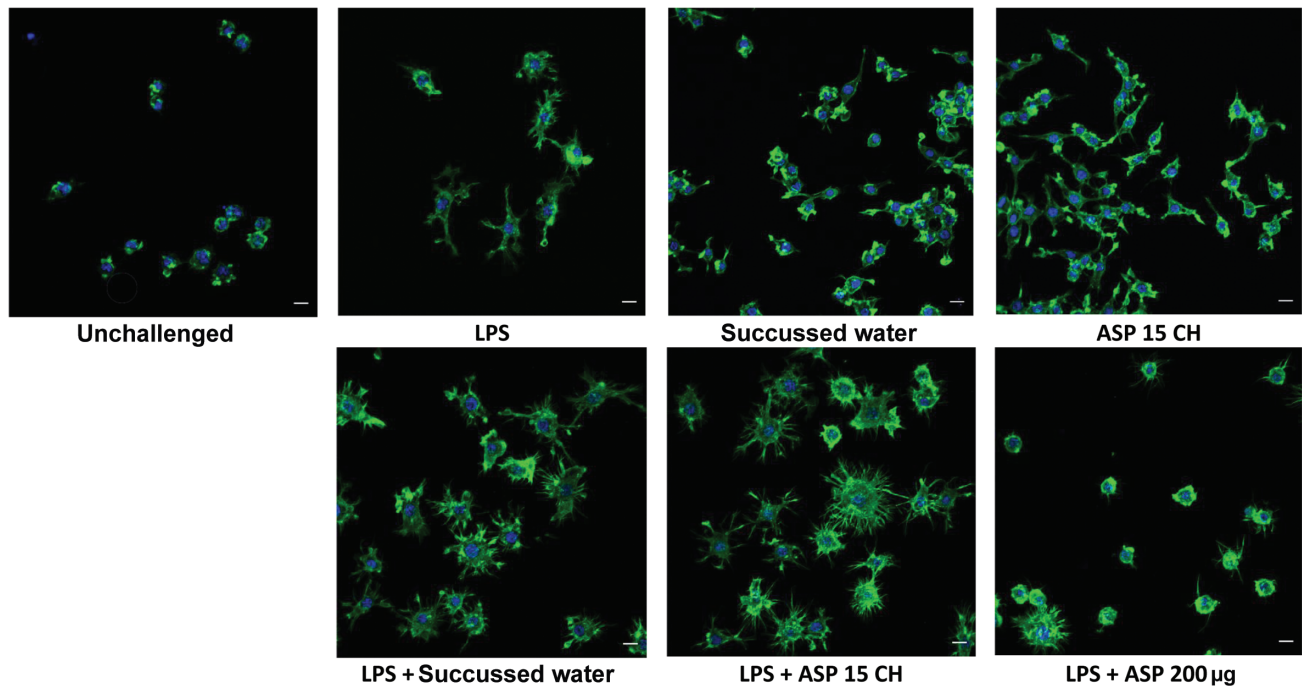
► Fig. 3 shows F-actin confocal microscopy of RAW 264.7 macrophages under different treatments. The main features can be described as follows: (1) unchallenged: round, non-activated cells with a large nucleus and small F-actin-labeled cytoplasmic extensions; (2) LPS: macrophages presenting increased cell area (spreading) and multi-directional long cytoplasmic extensions or pseudopods, labeled by F-actin filaments (light areas); (3) succussed water: round non-activated cells are seen together with cells presenting short bi-directional pseudopods delimited by F-actin filaments; (4) Aspirin 15cH: cells exhibiting medium and bi-directional pseudopods with dense F-actin labeled filaments in their extremities; (5) LPS + succussed water: cells presenting multiple short and multi-directional pseudopods intensely marked by the fluorescent dye following the expression of F-actin; (6) LPS + Aspirin 15cH: heterogeneous population of cells, some round with an intense expression of F-actin in the cytoplasm-membrane interface, others in a clear spreading process, presenting an increased adhered cell area and many multi-directional extensions with different lengths delimited by green, fluorescent stained F-actin filaments; and (7) LPS + aspirin 200  $\mu\text{g/mL}$ : mostly round macrophages showing almost no spreading, though presenting intense F-actin fluorescence.

In ►Supplementary Videos S1 to S7, different patterns of cell extensions (pseudopods) can be better noted in the 3D video. The spreading process after LPS treatment is bi- or multi-directional, with flat F-actin-labeled pseudopods following the slide surface (►Supplementary Video S2). As expected, this is different from the control (►Supplementary Video S1), in which almost no pseudopods are seen. However, cells treated with Aspirin 15cH show a particular feature in which the cytoplasm extensions assume a "columnar" shape, following a bi-directional pattern (►Supplementary Video S3). The combination of LPS and Aspirin 15cH treatments resulted in a heterogeneous cell population in which both patterns are seen, from round "columnar" cells to flat multi-directional cytoplasm extensions (►Supplementary Video S4). A few macrophages presenting a small number of short pseudopods are seen among round cells with "columnar" nuclei in samples treated with LPS and aspirin 200  $\mu\text{g/mL}$  (►Supplementary Video S5). A similar pattern is seen in succussed water-treated cells (►Supplementary Video S6). The LPS and succussed water combination produced multiple but short and thin multi-directional pseudopods in the macrophages (►Supplementary Video S7).



$F(6,68) = 19.85$ ,  $p \leq 0.0001$ ; Tukey,  $p \leq 0.03$  among categories

**Fig. 2** Cell area in pixels to demonstrate the RAW 264.7 macrophages spreading after different challenges and treatments. One way ANOVA,  $F_{(6,68)} = 19.85$ ,  $p = 0.0001$ ; Tukey test,  $p \leq 0.03$  among significance levels (a, b, and c). Data are represented by mean, standard deviation, and single data points. Two outliers were withdrawn.



**Fig. 3** F-actin confocal microscopy of RAW 264.7 macrophages under different treatments. For confocal microscopy, coverslips were stained with 2.5% phalloidin (green cytoplasmatic structures) with 10 µl of DAPI (blue nuclei). All images were registered under a 10x objective. F-actin appears in fluorescent cytoplasm contours.

### Cytotoxicity

No statistically significant difference ( $p=0.25$ ) among groups regarding cytotoxicity was seen when assessed by MTT assay. However, there was a trend for aspirin 200 µg/mL treatment to reduce the mitochondrial activity in LPS-activated cells (–**Supplementary Fig. S1**, available online only).

### Expression of TLR-4

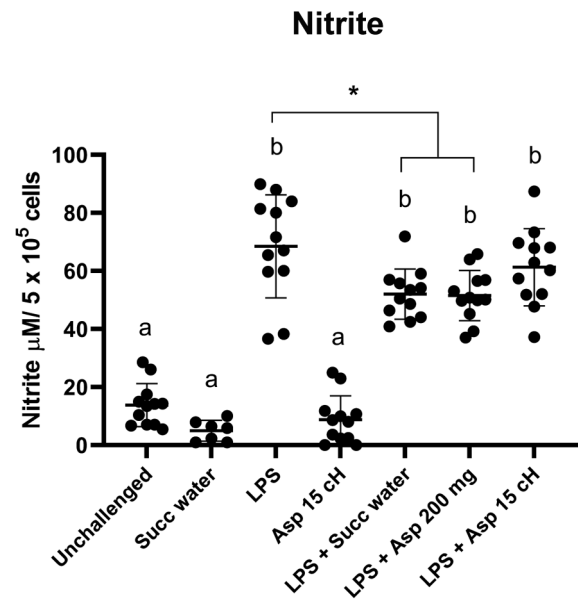
No significant difference in the number of TLR-4-positive cells was found between treatments, except for a statistically significant difference between LPS and Aspirin 15cH ( $p=0.03$ ), in which the treatment with Aspirin 15cH produced a clear and homogeneous decrease in TLR-4 expression (–**Fig. 1B**).

The MFI of TLR-4 labeling presented no significant differences among groups (–**Fig. 1C**).

### Nitrite Production

LPS challenge induced a consistent and significant increase in nitrite production ( $p < 0.0001$ ) in relation to unchallenged macrophages, regardless of any additional treatments. This result was useful to validate the Griess method used in this experiment since this effect related to LPS is already known.

Among the cells challenged with LPS, the co-treatment with succussed water and aspirin 200 µg/mL resulted in a small but significant reduction of nitrite production ( $p=0.007$ ) in relation to cells challenged with LPS only. The effects of Aspirin 15cH were comparable to those of succussed water, independent of LPS presence in the supernatant (–**Fig. 4**).



$F(6,72) = 66.60$ ,  $*p < 0.0001$ .  
Tukey,  $p < 0.0001$  among categories (a, b);  $*p = 0.007$

**Fig. 4** Nitrite ( $\mu\text{M}/5 \times 10^5$  cells) concentrations in supernatants of RAW 264.7 macrophage cultures submitted to different treatments and challenges. One-way ANOVA,  $F_{(6,72)} = 66.60$ ,  $p < 0.0001$ . Tukey test,  $p = 0.0001$  among categories (a, b);  $*p = 0.007$ . Data are represented by mean, standard deviation, and single data points. There were no outliers.

## Cytokines

Using the CBA mouse Inflammation® kit, levels of cytokine/chemokine production were quantified for IL-6, IL-10, MCP-1,  $\gamma$ -IFN, TNF- $\alpha$ , and IL-12 p70. No MCP-1 or IL-12 p70 was detected for any experimental groups, as levels of both were under the sensitivity limit of the method. No significant differences in levels of  $\gamma$ -IFN were detected among groups, though the ability of cells to produce this cytokine seems to be mainly related to the LPS and succussed water treatments ( $\blacktriangleright$  Fig. 5).

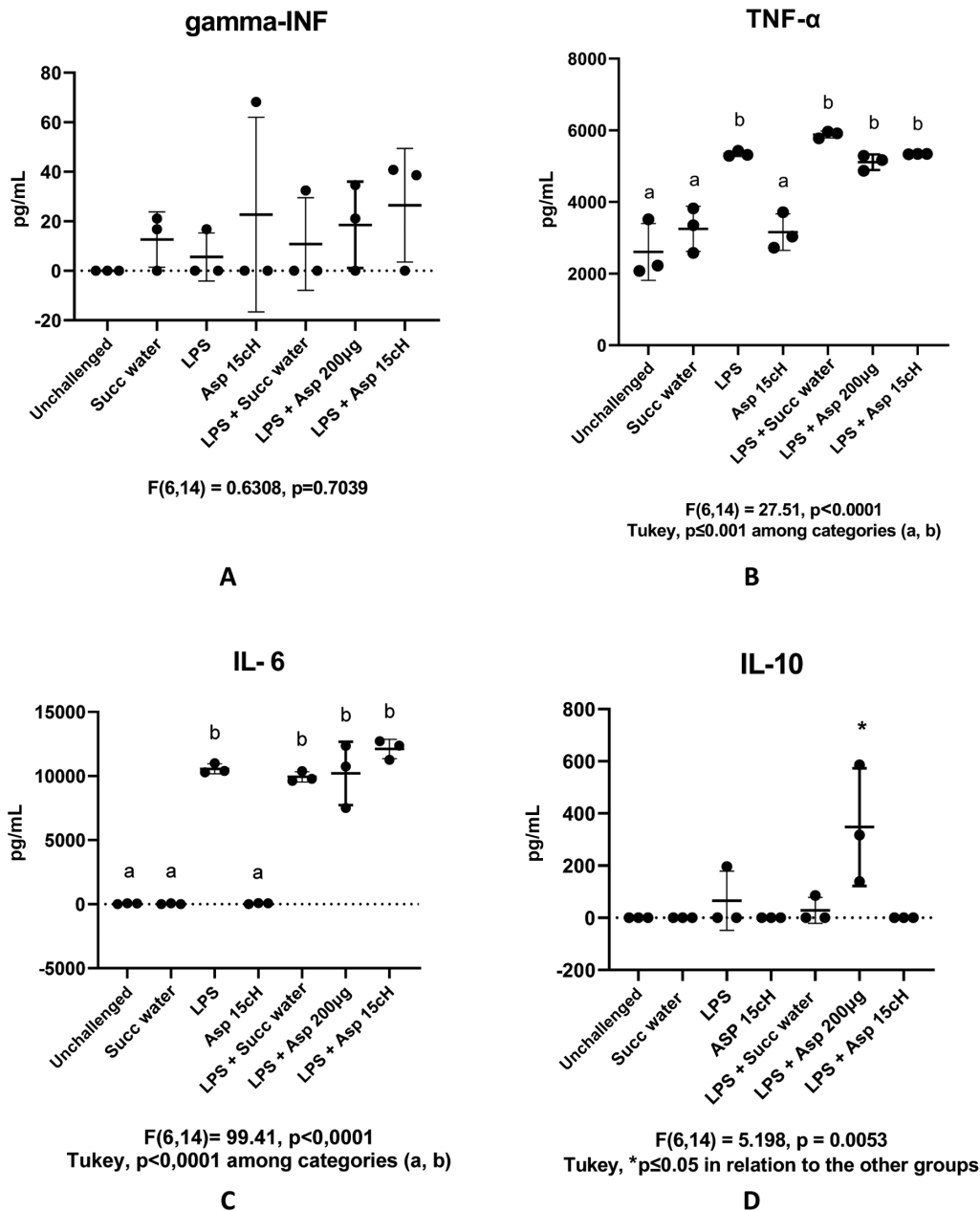
The high heterogeneity of the results is expected since the production of  $\gamma$ -IFN by macrophages is related to very specific conditions.<sup>31</sup> Moreover, IL-6 and TNF- $\alpha$  levels were

increased by challenge with LPS with or without co-treatments ( $p \leq 0.001$ ), and IL-10 production was increased only in the supernatants of cells treated with LPS + aspirin 200  $\mu$ g/mL ( $p \leq 0.05$ ) ( $\blacktriangleright$  Fig. 5).

These observations are consistent with the known pro-inflammatory action of LPS and the known anti-inflammatory effect of aspirin at a pharmacological concentration: succussed water and Aspirin 15cH had no effect on production of relevant cytokines.

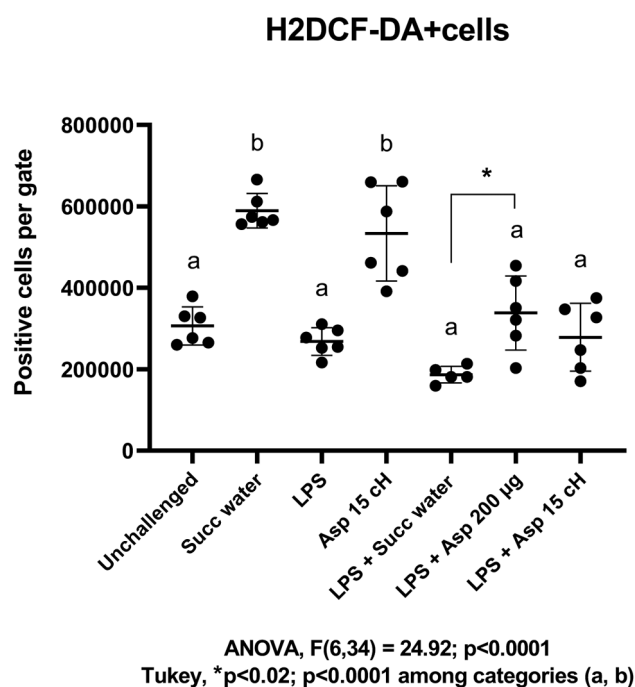
## Intracellular Reactive Oxygen Species Production

The number of cells producing ROS, as identified by flow cytometry, increased in macrophages treated with succussed



**Fig. 5** Cytokine concentrations (pg/mL) in supernatants of RAW 264.7 macrophage cultures submitted to different treatments and challenges. One-way ANOVA, following Tukey. (A)  $\gamma$ -INF,  $F(6,14) = 0.6308, p = 0.7039$ ; (B) TNF- $\alpha$ ,  $F(6,14) = 27.51, p < 0.0001$ ; Tukey,  $p \leq 0.001$  among categories (a, b); (C) IL-6,  $F(6,14) = 99.41, p < 0.0001$ ; Tukey,  $p < 0.0001$  among categories (a, b); (D) IL-10,  $F(6,14) = 5.198, p = 0.0053$ ; Tukey,  $*p \leq 0.05$  in relation to the other groups. Data are represented by mean, standard deviation and single data points. There were no outliers.





**Fig. 6** Number of positive cells to H2DCF-DA probe on the gate referring to species reactive to oxygen (ROS) in the intracellular space of RAW 264.7 macrophages submitted to different challenges and treatments. One way ANOVA,  $F_{(6,34)} = 24.92$ ,  $p < 0.0001$ . Tukey test,  $*p < 0.02$ ;  $p < 0.0001$  among categories (a, b). Data are represented by mean, standard deviation and single data points. There were no outliers.

water and *Aspirin* 15cH, suggesting a non-specific effect related to the succussed vehicle. However, this effect was lost in cells challenged with LPS, independent of any co-treatment. This reversion was less intense in cells treated with LPS and its inhibitor, aspirin 200 µg/ml, compared with cells treated with LPS and succussed water (► Fig. 6).

The MFI of ROS-labeling per cell did not differ significantly between groups (► **Supplementary Fig. S2**, available online only).

## Discussion

The primary motivation to study *Aspirin* 15cH in an LPS/macrophage model system *in vitro* arose from the previous work conducted by Doutremepuich et al.<sup>23,32</sup> and Eizayaga et al.<sup>24</sup> in which *Aspirin* 15cH presented thrombogenic features in different experimental situations. According to Eizayaga et al.<sup>24</sup> one hypothesis proposed was the possible inhibitory effect of *Aspirin* 15cH on the cyclooxygenase-2 (COX-2) activity involving local inflammatory cells. Indeed, in the former studies,<sup>23,24,32</sup> *Aspirin* 15cH was hypothesized to act via COX-2 inhibition, leading to prostaglandin PGE<sub>2</sub> decrease and thrombin stimulation, imposing a thrombogenic action in parallel with the modulation of the inflammatory process.<sup>33–35</sup> This proposition naturally leads to the following question: “What could be the effects of *Aspirin* 15cH on macrophage functions?”

The main aim of the present study was thus to explore what effects *Aspirin* 15cH might have on RAW 264.7 macro-

phages cultured *in vitro*. Exposure of cultured macrophages to *Aspirin* 15cH or LPS alone showed high statistical significance in terms of the effect on cell spreading (an increase of cell size when adhered to a glass surface) compared with the non-challenged cultures, treated or not with succussed water (vehicle). Surprisingly, combining *Aspirin* 15cH and LPS-challenge resulted in a null effect on adhered cell area, as if canceling each other out. This cancellation was also seen when LPS-challenge was associated with succussed water or its pharmacological blocker, aspirin 200 µg/ml.

*Aspirin* at 200 µg/mL was used as a positive control within the model system since its typical anti-inflammatory effects (by inhibiting COX-1 and COX-2) can block the LPS effects on macrophage spreading and increase IL-10 production,<sup>35,36</sup> as seen herein. However, it was not comparable with *Aspirin* 15cH effects, suggesting different mechanisms of action in each case.

*Aspirin* 15cH increased the average adhered cell area but exhibited bi-directional pseudopods with a “columnar” (high) shape, which was associated with reduction of the TLR-4 positive-cells population, mainly concerning LPS-treated cells. This finding led to the following explanatory hypothesis: the starting of an M2 polarization process (i.e., toward a wound healing/tissue repair phenotype, rather than the M1 inflammatory phenotype) in *Aspirin* 15cH-treated macrophages could be a possibility since the cell spreading, in this case, was characterized as long and tiny cytoplasmic pseudopods, with reduction of cell circularity besides the increase in cell perimeter, though with less F-actin production.<sup>37,38</sup> Often, these features are marked by the transcription of STAT6<sup>39</sup> and Arginase-1, which hydrolyzes arginine (an essential amino acid involved in NO synthesis during M1 polarization).<sup>40</sup> Further studies would be necessary to unveil the molecular paths involved in *Aspirin* 15cH stimulation of macrophages.

A paradoxical effect on cell morphology was seen when LPS and *Aspirin* 15cH treatments were used together. The average cell size was reduced in this case due to the heterogeneous population: some cells remained round, and others assumed a clear spreading process, with many multi-directional pseudopods with different lengths. The intense presence of F-actin in the pseudopod extremities showed the active process of F-actin polymerization, as expected in LPS-stimulated macrophages.<sup>41,42</sup> The activation and incorporation of F-actin monomers into macrophage podocytes are easily observed by specific staining,<sup>9,43</sup> in such a way that confocal microscopy can obtain representative images that enable quantitative image analysis.<sup>44</sup> Additionally, the average number of TLR-4-positive cells was intermediate in this case, with values between high expression (as seen in LPS-challenged cells) and low expression (as seen in *Aspirin* 15cH-treated cells), being comparable to the baseline. Together, these findings suggest the existence of two distinct cell populations whereby only one would be able to respond to *Aspirin* 15cH—possibly due to epigenetic or phenotypic modulation, since this kind of effect has previously been described in other cell models for studying the action of homeopathic formulations on macrophages *in vitro*.<sup>45–47</sup>

Besides F-actin, dynamic alteration of macrophage morphology depends on the rapid rearrangement of cytoskeletal microtubules in response to extracellular stimuli.<sup>48</sup> Bian et al demonstrated that stimulation of TLR-4 by LPS reduces the expression of  $\alpha$ - and  $\beta$ -tubulins, increasing microtubule plasticity and allowing changes in cell morphology.<sup>49</sup> Changes in microtubule stability induced by docetaxel and paclitaxel in homeopathic preparations have already been described,<sup>50</sup> suggesting that further studies of changes in the microtubule architecture of *Aspirin* 15cH-treated macrophages may be informative.

Some of the cell responses seen in this set of experiments were associated exclusively with the presence of LPS in the culture, independent of any co-treatment. These are the increased IL-6, TNF $\alpha$  and nitrite in the supernatants as well as the increased number of TLR-4-positive-cells.<sup>37,39</sup> Additionally, the increase of H2DCF-DA positive cells was seen in both groups treated with succussed vehicle (succussed water) and *Aspirin* 15cH, which suggests a non-specific effect probably related to the increase of cell oxidative activity following the presence of nano- and micro-bubbles generated during succussion.<sup>51–53</sup>

The mitochondrial activity measured in the MTT assay did not present any difference among groups, indicating no cytotoxic effects for any treatment. This is of particular importance when considering the morphology of the macrophages treated with aspirin 200  $\mu$ g/mL, succussed water, and *Aspirin* 15cH + LPS: a rounded morphology cannot be justified as being dead or degenerated cells, but rather non-activated.

The general expression of TLR-4 and intracellular ROS production, as evaluated by the MFI obtained by flow cytometry, did not change in response to any treatment. Instead, only the number of positive cells was associated with specific treatments. One could speculate that some stimuli could “wake up” specific cells presenting distinct phenotypes but not alter the average level of TLR-4 expression in the whole cell population. This speculation is consistent with the hypothesis of cell heterogeneity concerning *Aspirin* 15cH responsiveness postulated above. When taken as a whole, the results obtained lead to the clear conclusion that aspirin 200  $\mu$ g/mL and *Aspirin* 15cH work by different pathways.

It is known that even at concentrations beyond Avogadro's number ( $10^{-24}$  M), high dilutions of many drugs can modulate gene expression in biological systems, though in a non-linear manner.<sup>54–56</sup> From a more comprehensive point of view, the balance of functions in the different tested conditions combining the LPS challenge with varying kinds of aspirin preparations suggests the triggering of different levels of the hormesis process, which is one of the hallmarks of health and cell adaptation.<sup>57</sup> Indeed, the results demonstrated that *Aspirin* 15cH could induce the organization of macrophage pseudopods in a very particular way and reduced the cell contingent able to express TLR-4 on the cell surface, which is a phenotype compatible with M2 polarization.<sup>37,38</sup> On the contrary, aspirin 200  $\mu$ g/mL presented a classical effect related to M2 polarization by increasing the IL-10 expression, which was not achieved by *Aspirin* 15cH.<sup>58,59</sup>

As above, a paradoxical effect was seen when *Aspirin* 15cH and LPS were introduced in the culture, simultaneously reducing the average cell size. Nevertheless, the confocal images using fluorescent F-actin as a cytoskeleton marker revealed a clear heterogeneity of cell morphology. Some expressed a typical pattern of LPS stimulation, and others expressed a specific pattern of non-activated macrophages. This paradox suggests that different cell populations might respond preferably to LPS or *Aspirin* 15cH signals. Alternatively, the interaction of *Aspirin* 15cH and LPS could impair the full cell activation by LPS, leading to a partial macrophage polarization to M2 as opposed to the complete M2 polarization that is seen after treatment with aspirin 200  $\mu$ g/mL.

Additional epigenetic and gene expression modulation studies are needed to fully explore and test this finding. Targets would be the many factors related to M1/M2 polarization, such as TLR-4, CD-14, F-actin, microtubules, and other cytoskeleton components related to cell movement control.

## Conclusion

*Aspirin* 15cH, aspirin 200  $\mu$ g/mL, LPS, and succussed water were independent stimuli able to induce different patterns of macrophage response or inhibition, as seen by morphological and functional parameters. Aspirin 200  $\mu$ g/mL presented a classical anti-inflammatory effect by increasing IL-10 production. LPS produced the expected pro-inflammatory cell response, such as an increase in NO derivatives, IL-6, and TNF $\alpha$  production, together with raised numbers of TLR-4 positive cells, consistent with M1 polarization. Instead, *Aspirin* 15cH showed peculiar effects on macrophage morphology and TLR-4 expression, partially compatible with an M2 polarization process. Further studies on epigenetics and gene expression levels of cytoskeleton components and other M1-to-M2 polarization-related proteins are needed.

## Highlights

- The macrophage activity *in vitro* after *Aspirin* 15cH treatment was analyzed.
- *Aspirin* 15cH reduced the number of cells expressing TLR-4 on the surface.
- *Aspirin* 15cH induced a “columnar” cell morphology due to cytoskeleton rearrangement.
- Different cell sensitivity to LPS and *Aspirin* 15cH is postulated.
- *Aspirin* 15cH, aspirin 200  $\mu$ g/mL, LPS and succussed water appear to act by different intracellular pathways.

## Supplementary material

**Supplementary Videos S1–S7.** 3D video images showing different patterns of cell extensions (pseudopods).

**Supplementary Fig. S1.** MTT assay for cytotoxicity.

**Supplementary Fig. S2.** H2DCF-DA fluorescence intensity.

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## Conflict of Interest

None declared.

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