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MSC administration resolves experimental acute gout increasing specialized pro-resolving mediators synthesis through a super-induction of prostaglandin E_2

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Abstract

Background The resolution of inflammation is an active process triggered in the acute phase of inflammation and mainly directed by specialized pro-resolving mediators (SPMs). Modulating the inflammatory response in favor of resolution is a therapeutic strategy of enormous interest and value. Previous studies have shown that human adipose mesenchymal stem cells (MSCs) possess the ability to shorten the acute inflammatory response by hinting an early cyclooxygenase (COX)-2 induction. We studied the potential of MSC to accelerate the resolution of inflammation through the direct promotion of the local synthesis of SPMs, and the role of prostaglandin (PG) E_2 release in this process in a model of experimental acute arthritis.

Methods Gouty arthritis was induced in male New Zealand rabbits via intra-articular injection of monosodium urate (MSU) crystals. Human adipose-derived MSCs were administered systemically in a single dose. Synovial membrane levels of SPMs were measured by liquid chromatography–tandem mass spectrometry, PGE_2 and genes related to the pro-resolving and anti-inflammatory pathways were assessed. Employing THP-1-derived macrophages and human adipose-derived MSC co-cultures stimulated with MSU crystals, we elucidated the mechanisms associated to the induction of resolution by MSCs.

Results MSC treatment enhanced the local release of a broad range of SPM precursors and active mediators in the synovial membrane of rabbits with gouty arthritis. This release occurred simultaneously with an early increase in PGE_2 levels, and an upregulation of COX-2 and the PGE_2 receptor EP4. Additionally, an early anti-inflammatory gene response was observed, characterized by increased expression of IL-10, indoleamine 2,3-dioxygenase-1 (IDO-1), and Formyl Peptide Receptor2 (FPR2). In cell culture experiments, we confirmed that MSCs are responsible for the release of pro-resolving and anti-inflammatory mediators, promoting macrophage efferocytosis and polarization towards a pro-resolving and anti-inflammatory M2 phenotype in a COX-2- and FPR2-dependent manner.

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Conclusions MSCs exerted a pro-resolving effect on the synovial membrane in gouty arthritis. This therapeutic action may be driven by an early superinduction of local PGE_2 synthesis and the promotion of a pro-resolving and anti-inflammatory M2 macrophage phenotype via COX-2 signaling and involving FPR2.

Keywords Specialized-pro-resolving mediators (SPMs), Resolution, Acute inflammation, PGE2, COX-2, Macrophages, Arthritis

Background

The resolution of inflammation is the process that actively terminates the acute inflammatory response in a controlled manner, ultimately allowing for the *ad integrum* repair of the affected tissue and preventing the progression to chronic inflammation. Despite remarkable advances in the field, the understanding of the resolution process is still limited, as does our knowledge of how anti-inflammatory treatments influence these processes [1, 2].

Resolution of inflammation orchestrates key mechanisms of the acute innate immune response. Gout, the most common type of acute inflammatory arthritis in adults, represents a paradigm of self-resolving acute inflammation, providing a unique clinical model to study pro-resolving mechanisms in a well-defined context. In human gout flares, the primary inducing agent is the deposition of monosodium urate (MSU) microcrystals in the joint, that activates the innate immune response in the tissue [3]. The inflammatory cellular response is triggered by an increase in the concentration of different proinflammatory lipids, such as leukotriene (LT) B_4 and prostaglandin (PG) E_2 in the synovial fluid, both derived from arachidonic acid (AA) [4–6]. These mediators precede the local recruitment of inflammatory cells and the upregulation of pro-inflammatory cytokines. The resolution phase is initiated when, within the inflamed environment, there is a shift towards the synthesis of anti-inflammatory and pro-resolutive mediators surpassing that of inflammatory cytokines [7, 8].

PGE_2 plays a dual role: while it contributes to the initiation of inflammation, it also plays a central role in driving the anti-inflammatory response, as suggested by our work in acute gout and by that of others who explore the cellular and molecular events governing tissue repair in acute inflammation [9–11]. In addition to inducing the expression of anti-inflammatory mediators such as IL-10 and indoleamine 2,3-dioxygenase (IDO) [12–14], PGE_2 may also regulate the switch from pro-inflammatory to pro-resolving lipid mediator synthesis by modulating the activity of different lipoxygenases (LOX) in inflamed tissue [7, 10, 15]. Through specialized pro-resolving mediators (SPMs), neutrophils and macrophages receive and amplify "inflammation stop" signals and promote inflammation resolution. This phase comprises the active blockade of blood cell infiltration, the induction of neutrophil apoptosis, the activation of non-phlogistic efferocytosis in macrophages, as well as the release of growth factors that eventually promote the repair of damaged tissue [12–14]. SPMs, mainly lipids in nature, are synthesized through the activity of LOX which use AA or omega-3 fatty acids such as docosahexaenoic or eicosapentaenoic as substrate to the release of different mediators that constitute four main families: lipoxins (LX), resolvins (Rv), maresins (Mar), and protectins (P) [16–18]. Levy and co-workers demonstrated that the increase in PGE_2 concentration preceded the resolution of the inflammatory event and the synthesis of SPMs in a mouse air-pouch model [7]. In a model of tail amputation in zebrafish, PGE_2 promoted the resolution of inflammation inducing an anti-inflammatory and pro-resolving phenotype in neutrophils, favouring the synthesis of SPMs, including LXA_4 [10]. For decades, anti-inflammatory therapies have primarily focused on suppressing specific pro-inflammatory mediators to alleviate symptoms and reduce the immune response. However, this approach often overlooks potential side effects and fails to address the whole inflammatory process on its complexity.

Accumulating evidence shows that mesenchymal stem cells (MSCs) foster tissue repair through the regulation of the inflammatory response and the promotion of tissue regeneration due to their potent immunomodulatory and reparative properties [19]. However, the anti-inflammatory capacity of MSCs is not intrinsic, but rather context dependent. Their behaviour is shaped by the surrounding tissue environment, allowing them to exert either pro- or anti-inflammatory effects depending on local conditions [19, 20]. In any case, the presence of different innate immunity stimuli or pro-inflammatory mediators induces MSCs to synthesize soluble factors that trigger an anti-inflammatory net response both in the MSCs and in surrounding cells [19]. A critical aspect of MSC therapy is their dynamic interaction with macrophages [9, 21]. MSCs induce macrophage polarization toward an anti-inflammatory M2 phenotype by secreting soluble mediators such as PGE_2, growth factors, and other paracrine mediators [9, 21]. However, this property is still relatively underexplored and poorly characterized in clinical and preclinical settings, particularly in acute inflammatory conditions [22, 23]. M2 macrophage polarization modulates the inflammatory response and promotes tissue repair. Furthermore, the communication between MSCs and macrophages is bidirectional, creating a feedback loop that amplifies the immunoregulatory

effects of MSCs and positions them as central regulators of cytokine storms in inflammatory pathologies [24].

We have recently demonstrated that systemic MSC administration ameliorate experimental acute gouty arthritis through an early induction of COX-2 [11], setting one of the main negative feedback of early control of acute inflammation [9, 25]. We observed that an early MSC-mediated super-induction of PGE_2 release in the synovial membrane (SM) preceded macrophage polarization towards M2 phenotype, the recovery of damaged synovium, the inhibition of nuclear factor (NF)κB and NOD-like receptor family pyrin domain containing (NLRP)3 inflammasome activation, and the downregulation of key proinflammatory cytokines such as IL-1β in the SM [11]. Similar data have been recently published in a mice model of heart damage [26]. However, whether MSCs can directly influence local SPM production through PGE_2 induction as a mechanism to modulate resolution in vivo remains poorly described.

Therefore, we aimed to investigate whether MSC administration triggers an accelerated resolution of gouty arthritis flare through the local synthesis of SPMs, and its relationship with PGE_2 levels in the SM of arthritic rabbits. Additionally, we explored the mechanisms by which PGE_2 regulates these processes in THP-1-derived macrophages stimulated with MSU microcrystals.

Materials and methods

Experimental model of acute gouty arthritis

Gouty arthritis was induced in three-month-old male New Zealand white rabbits (weight 2.5–3.0 kg, Granja San Bernardo, Spain) as previously described [11, 27, 28]. Animals were housed individually in cages that provided 0.50 m of cage height (0.6 m^2 of floor space) in rooms with 12 daily hours of light. After 15 days of acclimatation, 32 anesthetized rabbits received an intra-articular injection of 50 mg of MSU crystals (resuspended in 1 ml of PBS) into each knee. One hour after MSU injection, 16 of these rabbits received a single dose of 2×10^6 MSC/kg via the central ear artery, resuspended in 2 ml of PBS (MSU + MSC group), while 16 rabbits received PBS using the same procedure (MSU group). We simultaneously tracked 8 sex- and age-matched rabbits that were injected with 1 ml of PBS in both knee joints and served as controls.

A single systemic dose of 2×10^6 MSC/kg was selected based on our previous rabbit MSU-arthritis study using the same dose, which demonstrated accelerated resolution and COX-2/PGE_2 induction [11]. This dose also falls within the $1–2 \times 10^6$ MSC/kg range most frequently used in clinical trials, ensuring translational relevance while avoiding non-linear or adverse effects observed at higher doses in pilot titrations (unpublished data). MSC were isolated from the adipose tissue from human healthy donor lipoaspirates after informed consent according to institutional guidelines and the approval by the Ethics Committee of Hospital Fundación Jiménez Díaz. MSC were obtained and characterized as described previously [11, 29].

In order to eliminate bias, blinding was performed by using a non-consecutive numerical code for each animal. The scientist in charge of preparing the intra-articular injection (MSU or saline) was different than the one performing the injections, and the same applies for the administering of MSC or PBS. In addition, the outcomes were assessed blinded to group assignment [11, 30].

Eight rabbits from MSU and eight from MSU + MSC group were euthanized by intracardiac injection of pentobarbital (50 mg/kg, Braun Medical SA, Spain) 24 h after MSU injection, while the remaining animals were euthanized 72 h after MSU administration. SM from both knees were then collected and processed for further studies. These tissues were immediately frozen in liquid N_2 for mass spectrometry (left knees) and molecular biology studies (right knees). Samples were collected at 24 h and 72 h after MSU injection to interrogate the canonical peak (24 h) and early-resolution (72 h) phases of acute gouty inflammation in rabbits, consistent with clinical and experimental flare kinetics [11].

All the experiments were performed in accordance with the Animal Research Reporting of In Vivo Experiments (ARRIVE 2.0) guidelines and with the National regulation and the Guidelines for the Care and Use of Laboratory Animals, drawn up by the National Institutes of Health (Bethesda, MS, USA). These procedures were approved by the Institutional Ethics and Animal Welfare Committee of IIS-FJD. According to the approved protocol, humane endpoint criteria for animal euthanasia were defined a priori as follows: severe impairment of mobility, posture, or behaviour; weight loss exceeding 20% during the study; and severe self-injury. Each criterion was scored from 0 (normal) to 3 (severe). Animals were daily checked and were euthanized if they reached a score of 3 in any single category or in the combined total.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The SM concentration of 23 lipid mediators was determined by LC-MS/MS: ω-3 polyunsaturated fatty acids (PUFAs): eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA); their monohydroxylated mediators: 18- hydroxyeicosapentaenoic acid (18-HEPE), 17- hydroxy DHA acid (17-HDHA), and 14-HDHA; AA and its derivatives: prostaglandins PGD_2 and $PGF_{2\alpha}$; and LXA_4, LXB_4, and LTB_4; thromboxane (TX)B_2; and SPMs including Rvs from EPA and DHA: RvE1, RvD1, RvD2, RvD3, RvD4, and RvD5; Mars and Ps from DHA: Mar1, Mar2, PD1, and PDX. Lipid mediators

were extracted from 24- and 72-hour rabbit SM by using a solid-phase extraction (SPE) method. Each sample (100 mg tissue) stored at − 80 °C was thawed on ice. Internal labelled standards d8-5-HETE, d5-RvD2, d5-LXA4, d4-LTB4, and d4-PGE2 (500 pg. each, Cayman Chemical) in 4 ml of methanol (Methanol Optima LC/MS Grade, Fisher Chemical) were added to each sample. Known concentrations of lipids in labelled standards were used for quantification purposes and posterior calculations on their recovery. Calibration curves were performed using synthetic and authentic lipid mediator mixtures, including d4-LTB_4, d5-LXA_4, d4-PGE_2, d5-RvD2, 5(S)-HETE-d8, RvD1, RvD2, RvD3, RvD4, RvD5, PD1, PDX, Mar-1, Mar-2, RvE1, LXA4, LXB4, PGD_2, $PGF_2\alpha$, TXB2, and LTB_4 at 1, 5, 25, 50, 100, and 200 pg. Linear calibration curves for each compound were obtained with R^2 values between 0.993 and 0.999. Then, the samples were placed at − 80 °C for 30 min for protein precipitation and separation by centrifugation (2000×g, 10 min, 4 °C). SPE was performed according to reported methods [31, 32]. Furthermore, samples were quickly acidified to pH = 3.5 with 9 ml of acidic water (HCl) just prior to loading onto SPE columns (100 mg, 10 ml, Biotage) and pH neutralization with 4 ml of Milli-Q water, followed by a washing step with 4 ml of n-hexane. Compounds were then eluted with 9 ml of methyl-formate. Extracts from the SPE were dried under a gentle stream of N_2 and immediately resuspended in methanol/water (50 : 50 vol/vol) before injection into an LC–MS/MS system [31, 32].

Measurement of PGE_2 concentration

One milligram of frozen SM was powdered and 1 mL of 1X PBS + 0.1 mM EDTA (Sigma-Aldrich) was added. In addition, each sample was subjected to three homogenisation cycles, 10 s each, in the Polytron LabGen 125 homogeniser (Cole-Parmer) with 10 min ice-rest intervals between cycles. One hundred µL of each extract was used for PGE_2 quantification (Cayman Chemical) by ELISA according to the manufacturer's instructions.

Co-culture of THP-1-derived macrophages and MSCs

THP-1 monocyte cells (passage 5 to 7) (American Type Culture Collection) were grown at 37 °C and 5% CO_2 in RPMI 1640 (Gibco BRL) supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin and 1% L-Glutamine (Gibco BRL). THP-1 monocytes were differentiated to macrophages in the presence of 0.5 µM Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 3 h. 1×10^6 THP-1 cells were seeded in 6 well plates and incubated O/N at 37 °C and 5% CO_2 in fresh medium RPMI 1640, 2% heat-inactivated FBS, 1% penicillin-streptomycin and 1% L-Glutamine. 2.5×10^5 MSCs (passage 3–4) were seeded and incubated into polycarbonate Transwell inserts of 24 mm and 0.4 µm membrane pore size (Corning) O/N in RPMI 1640, 2% heat-inactivated FBS, 1% penicillin-streptomycin and 1% L-Glutamine. PMA-differentiated THP-1 macrophages were stimulated with MSU crystals (Invivogen) in a concentration of 150 µg/mL or vehicle (PBS). One hour after the addition of the crystals, Transwell inserts were placed over THP-1 macrophages in a ratio 1:5 (MSC: THP1) [11]. Cells were co-cultured during 24 h after MSU addition.

To investigate the role of PGE_2 exclusively synthesized by MSC, we performed a separate incubation of these cells with the COX-2 specific inhibitor NS-398 (5 µM, Sigma-Aldrich) for 24 h. Then, the media was removed and MSC were added to the THP-1 co-culture. Where indicated, the following substances were added to the co-culture media simultaneously with MSU crystals: PF04418948 (1 µM, Tocris Bioscience), an antagonist of the PGE_2 EP2 receptor; L-161,982 (1 µM, Tocris Bioscience), an antagonist of the PGE_2 EP4 receptor; or WRW4 (10 µM, Tocris Bioscience), an antagonist of the N-formyl peptide receptor 2 (FPR2) which mediates the pro-resolving effects of LXA_4 and selected SPMs.

Assessment of efferocytosis, polarization markers and soluble mediators in THP-1-derived macrophages

To efficiently study the gene expression and release of different markers of efferocytosis or polarization, THP-1-derived macrophages cells were differentiated to M1 phenotype. One million THP-1 monocytes were seeded in p6 well plates (Corning) and 100 nM PMA was added for 24 h, and cells were left to rest for another 24 h. Then, cells were incubated with 100 ng/mL LPS plus 20 ng/mL IFN-γ for 24 h, when MSU crystals were incorporated. One hour after MSU addition, MSCs in transwell were added to the co-culture. Where indicated, COX-2 specific inhibition in MSC was performed as indicated above, or a FPR2 antagonist was added in the co-culture medium.

RNA isolation and real time-PCR studies

RNA was isolated employing TRIzol reagent (Roche Diagnostics), dissolved in nuclease free water and quantified [30, 33]. Retro-transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. Gene expression was quantified by single-reporter real-time PCR employing the Step One Plus Detection system (Applied Biosystems) and the following TaqMan probes listed: ALOX12 (gene encoding for 12-LOX), ALOX15 (gene encoding for 15-LOX), ALOX5 (gene encoding for 5-LOX), arginase (ARG)1, CD163, CD206, CD36, CD68, CD80, COX-2, PGE_2 receptor (EP)1, EP2, EP3, EP4, FPR2, G protein-coupled receptor 32 (GPR32), IDO-1, IL-10, IL-1β, Mer Tyrosine Kinase (MerTK), Prostaglandin E Synthase (PTGES) and Tumour Necrosis Factor-Stimulated Gene 6 (TSG6) (all

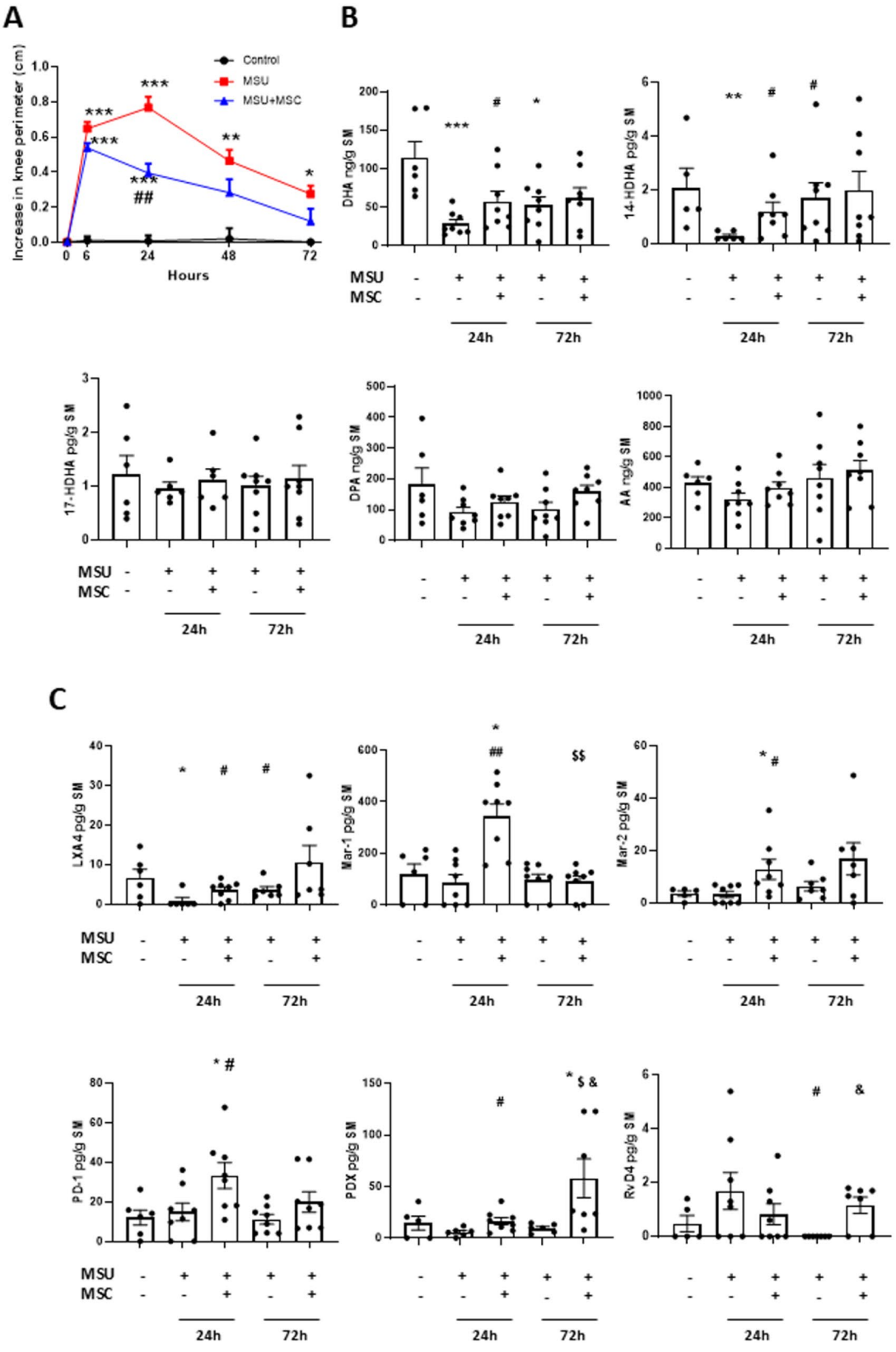

Fig. 1 (See legend on next page.)

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Fig. 1 Systemic administration of MSC through the central ear artery prompted a pro-resolving response in the SM from rabbit knees injected with MSU crystals. **A** Effect of MSC administration on the knee perimeter along the study. Each data point shows the mean and SEM, $n = 8$ for all groups. **B** LC-MS/MS quantification of SPM and prostanoid precursors in synovial membrane. Bars show the mean and SEM; $n = 6$ for Control group; $n = 8$ for MSU and MSU + MSC groups for each time point studied. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ vs. Control; #$p < 0.05$, ##$p < 0.001$, ###$p < 0.0001$ vs. MSU 24 h; $$p < 0.05$ vs. MSU + MSC 24 h; &$p < 0.05$ vs. MSU 72 h. 14-HDHA: 14-hydroxy docosahexaenoic acid; 17-HDHA: 17-hydroxy docosahexaenoic acid; AA: arachidonic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; LXA4: Lipoxin A4; Mar-1: Maresin-1; Mar-2: Maresin-2; MSC: mesenchymal stem cells; MSU: monosodium urate; PD-1: Protectin D1; PDX: Protectin DX; RvD4: Resolvin D4

from Thermo Fisher Scientific). Quantitative measurements were determined using the ΔΔCt method and expressed as fold change. Gene expression of HPRT was used as the internal control [11, 30].

Western blot studies

Total proteins were extracted separately from rabbit SM, THP-1 macrophages or from MSC lysates, resolved on SDS-PAGE gels and transferred to nitrocellulose membranes in a semi-dry Trans-Blot device (Bio-Rad) [30]. The following primary antibodies were applied overnight at 4 °C: anti-human COX-2 1:1000 (Thermo Fisher Scientific); anti-human LOX-5 1:1000 (BD Biosciences); anti-rabbit LOX-12 1:500 (Abcam); anti-mouse LOX-15 1:500 (Abcam); and anti-human ARG-1 1:5000 (Proteintech). Protein loading control was performed employing Coomasie-blue staining for rabbit tissue samples [33], and β-actin 1:10000 (Cell Signaling) for isolated cell samples.

MerTK immunofluorescence

One million THP-1 were seeded in 2 mL of 10% RPMI, differentiated to M1 as described above, and co-culture in transwell with MSC. After 24 h of incubation with 150 ug/mL MSU crystals, cells were fixed with 4% PFA and incubated with 5% BSA for 1 h. An anti-MerTK (Abcam) was used as the primary antibody (1:100), and the anti-rabbit-Alexa fluor 488 antibody (1:200, Invitrogen) was employed for detection. Cells were counterstained with DAPI (Thermo Fisher Scientific), and preparations were mounted in Vectashield Mount Medium (Merck).

ELISA measurements in culture media

Supernatants were collected from cocultures between MSCs and THP-1. They were centrifuged at 4° for 10 min at 2000 g to remove MSU crystals. These samples were later analysed according to the manufacturer's instructions for PGE_2 (Cayman Chemical), LXA_4 (Cayman Chemical), RvD1 (Cayman Chemical), IL-10 (Enzo) and IL-1β (Abcam).

Efferocytosis assay

Fifty thousand THP-1 cells were polarized to M1 macrophages in a p96 black plate. Direct coculture was performed between MSC and M1 THP-1 in a ratio 1:10 (MSC: THP1). For 24 h, 75 µg/mL MSU were added in the presence or absence of 5 µM NS-398 or 10 µM WRW4. Finally, fluorescent red *E.coli* bioparticles were added according to manufacturer's instructions (Invitrogen, MA, USA) for 1 h at 37 °C. Data were collected at this time.

For confocal imaging, 1×10^6 THP-1 were seeded on top in 2 mL of RPMI 1640 10% FBS medium and differentiated to M1 and MSC were directly added as previously described. Then, pHrodo was added and incubated for 3 h at 37 °C. Then, cells were fixed with 4% PFA for 15 min and counterstained with DAPI (Thermo Fisher Scientific).

Statistics

Statistical analysis was performed with the GraphPad Prism software suite (8.0.2 for Windows). A ROUT outlier test (Q = 5%) was applied to all experiments. Data were tested for normality. Parametric tests were used when these assumptions were met; otherwise, non-parametric tests were applied. ANOVA test followed by *post hoc* Bonferroni correction was performed to compare three or more variables (One-Way ANOVA) and for the development of joint perimeter between different groups (Two-Way ANOVA). For comparison between two variables, a non-parametric Mann-Whitney test was performed. Quantitative data are expressed as mean ± SEM. P values less than 0.05 were considered significant. For the population size calculation of the gouty arthritis model treated with MSC, previous data of PGE_2 levels in SM [11] gave us a sample size of 8 rabbits per group, with a power of 95%, $p = 0.05$. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology.

Results

Modulation of the synthesis of different SPM and their precursors by MSC in the SM of arthritic rabbits

As shown in Fig. 1A, MSU + MSC animals showed an early reduction in joint perimeter compared to MSU group. Similar results were described in a model where MSC was administered via the femoral artery [11]. According to this finding, the treatment induced a less intense and shorter inflammatory gouty flare. MSU + MSC animals exhibited a joint perimeter similar to controls at 72 h, while joint swelling remained significantly higher in the MSU group (Fig. 1A). Therefore, we decided to investigate the effect of MSC administration on the presence of SPMs in the SM at 24 and 72 h following MSU injection.

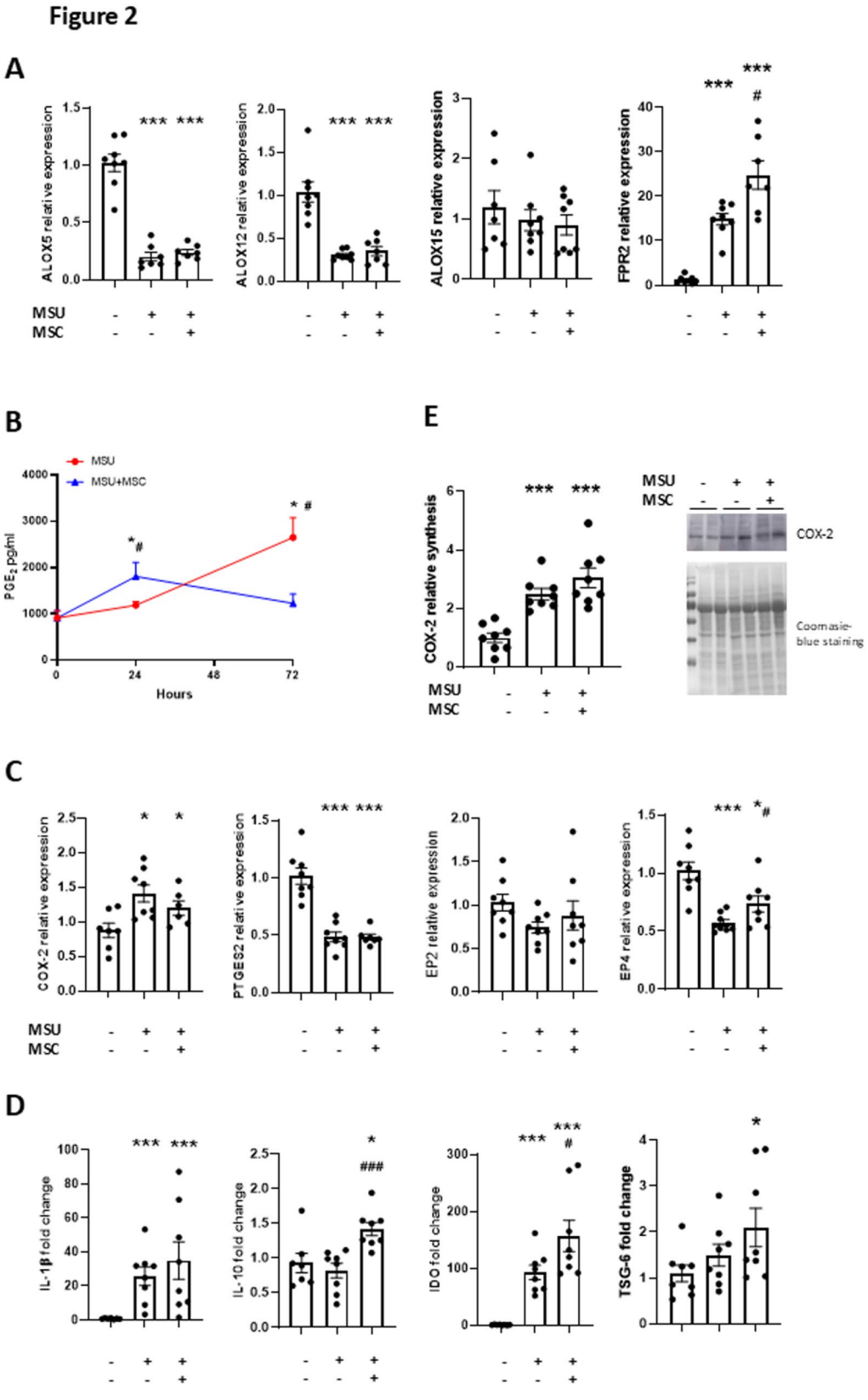

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Fig. 2 **A** Lipoxygenases genes and pro-resolutive FPR2 receptor gene expression in the synovial membrane of the rabbits 24 h after MSU injection. Bars show the mean and SEM **B** PGE_2 concentration along 72 h in the synovial membrane of the rabbits. Each data point represents the mean and SEM. **C** COX-2 pathway and EP receptors gene expression in the synovial membrane of the rabbits 24 h after MSU injection. Bars show the mean and SEM **D** Pro- and anti-inflammatory mediators gene expression in the synovial membrane of the rabbits 24 h after MSU injection. Bars show the mean and SEM. **E** COX-2 protein presence in the synovial membrane of the rabbits 24 h after MSU injection. Bars show the mean and SEM. Representative western-blot images are shown, together with the corresponding loading controls. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ vs. Control; #$p < 0.05$, ##$p < 0.001$, ###$p < 0.0001$ vs. MSU. $n = 8$ for each group, for each time point studied. ALOX-5: arachidonate 5-lypoxigenase; ALOX-12: arachidonate 12-lypoxigenase; ALOX-15: arachidonate 15-lypoxigenase; COX-2: cyclooxygenase-2; FPR2: formyl peptide receptor 2; EP: prostaglandin E_2 receptor; IDO: indoleamine 2,3-dioxygenase-1; IL: interleukin; PTGES2: prostaglandin E synthase 2; TSG-6: TNF alpha induced protein 6

MSU administration induced a significant decrease in the levels of docosahexaenoic acid (DHA) and 14-hydroxydocosahexaenoic acid (14-HDHA) --both precursors of D-series Rv, PD and Mar) -- at 24 h compared to control animals. This decrease remained significant at 72 h for DHA (Fig. 1B). However, MSU intraarticular injection did not modify other SPM precursors such as 17-hydroxydocosahexaenoic acid (17-HDHA), docosapentaenoic acid (DPA), or AA, in comparison to control animals (Fig. 1B). In the MSU group, we also observed a significant decrease in LXA_4 concentration at 24 h, while no significant changes were observed for Mar-1, Mar-2, PD-1, PD-X in comparison to controls (Fig. 1C). Several of the lipid mediators included in the analysis were below the detection limit in most of the SM samples; consequently, these data are not shown.

At 24 h, MSC administration significantly increased DHA and 14-HDHA levels compared to the MSU group (Fig. 1B). In addition, MSCs induced an early synthesis of the SPMs LXA_4, Mar-1, Mar-2, PD-1 and PD-X, compared to the MSU group (Fig. 1C). At 72 h, the presence of PD-X and RvD4 remained significantly higher in MSU + MSC group compared to the MSU, while the rest of the SPMs returned to control values, except for PD-1 (Fig. 1C).

In summary, MSU injection decreased DHA and 14-HDHA levels and reduced LXA4 concentration, whereas MSC administration restored precursor levels and increased the synthesis of several SPMs at early time points.

Effect of MSC treatment in the early regulation of LOX, COX-2 and anti-inflammatory pathways in the SM of arthritic rabbits

Our data show a significant decrease in the gene expression of ALOX5 and ALOX12 in the SM of MSU and MSU + MSC groups in comparison to Control at 24 h. No differences were found in ALOX15 expression (Fig. 2A). Furthermore, the shared SPM receptor FPR2 was significantly increased in the arthritic groups, in comparison to Control at 24 h. MSC administration induced a further increase in the expression of this receptor compared to the MSU group (Fig. 2A).

We showed that MSU + MSC group had a higher PGE_2 concentration in the SM in comparison to MSU group at 24 h, coinciding with the increase in the presence of pro-resolutive mediators in the tissue. (Fig. 2B). However, at 72 h, PGE_2 levels were significantly decreased in the MSU + MSC group, in line with the decrease in joint oedema (Fig. 2B).

Regarding the enzymes involved in PGE_2 synthesis in the SM, we observed an increase in COX-2 expression in the MSU and MSU + MSC groups, vs. the Control group at 24 h (Fig. 2C). However, PTGES2, was significantly decreased in the MSU and MSU + MSC groups compared to the Control group (Fig. 2C). No differences were observed in the gene expression of these enzymes between MSU and MSU + MSC groups. As for the receptors involved in the anti-inflammatory PGE_2 response, we did not observe any change in EP2 expression between groups 24 h after crystal injections. A significant decrease in EP4 levels in MSU and MSU + MSC was shown. However, a significant increase in the MSU + MSC group compared to the MSU group (Fig. 2C) was observed. Therefore, in the MUS-MSC group there was a transient increase in PGE_2 levels and a modulation in EP4 receptor expression without affecting COX-2 or PTGES2 expression compared to the MSU group.

Finally, we decided to assess the early effect of MSC administration on the modulation of different pro and anti-inflammatory mediators in the SM. As shown in Fig. 2D, an increase in the synthesis of IL-1β in the MSU and MSU + MSC vs. Control groups was observed. MSC administration did not showed any significant effect in comparison to MSU group. Regarding the levels of the anti-inflammatory cytokine IL-10, no variation was observed in the MSU group. MSC administration promoted its synthesis, both in comparison to MSU and to Controls (Fig. 2D). In addition, IDO-1 upregulation occurred in both arthritic groups compared to the Control. A significantly higher increase was observed in the MSU + MSC group in comparison to the MSU group (Fig. 2D). Finally, although TSG6 gene expression was not significantly different between MSU and MSU + MSC groups, a significant increased expression in the MSU + MSC group was observed vs. Control (Fig. 2D).

COX-2 protein levels were also evaluated to confirm the gene expression results. As shown in Fig. 2E, the protein data mirrored the transcriptional findings, with a significant increase in synovial membrane COX-2 levels

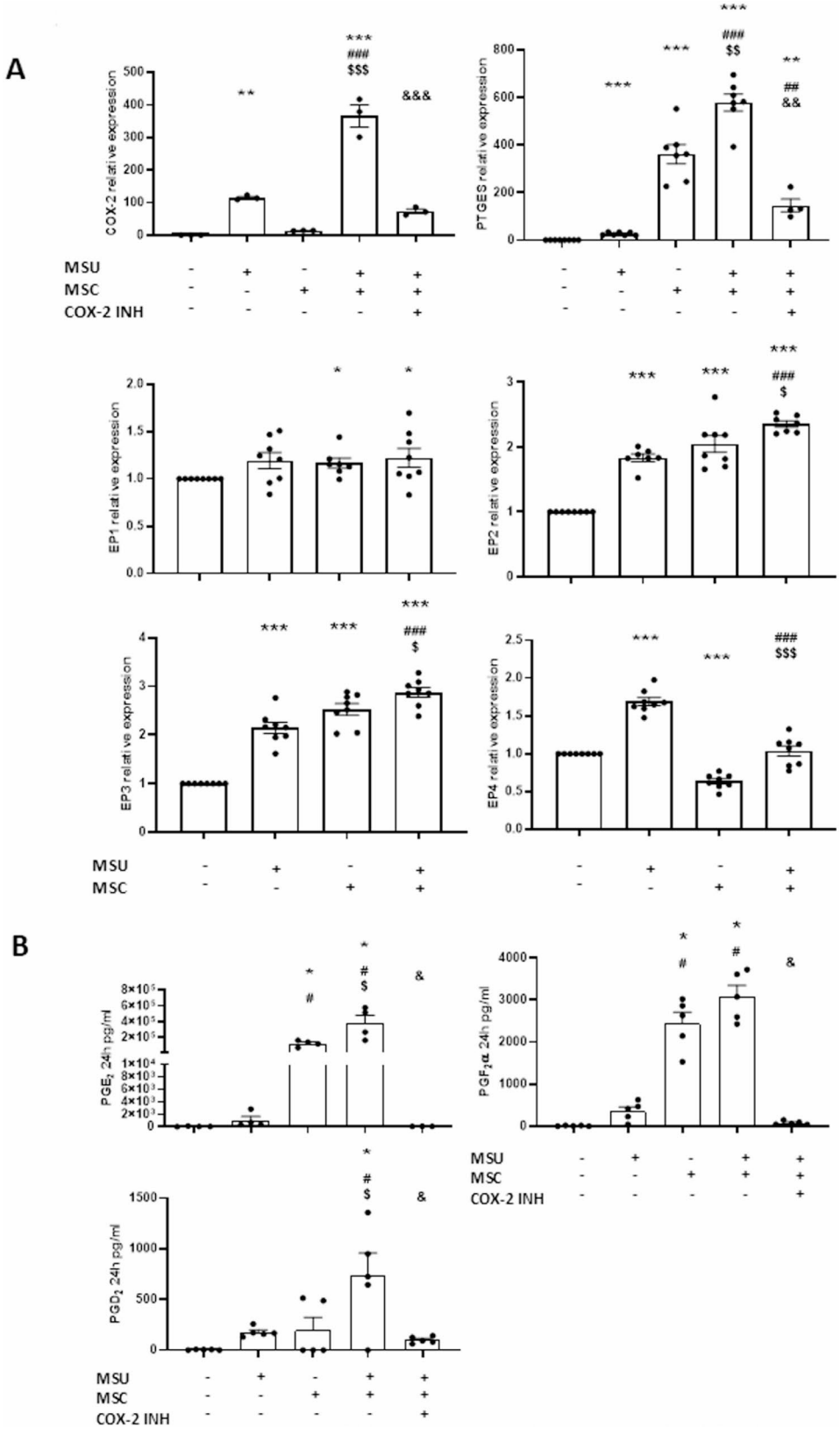

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Fig. 3 MSC addition activated COX-2 pathway and EP receptors in THP-1 macrophages and was responsible for the increase in prostaglandin synthesis. **A** Gene expression of COX-2, PTGES and EP receptors in THP-1 macrophages. **B** Different prostaglandins levels in MSC-THP-1 co-culture media in the presence or absence of the COX-2 inhibitor NS-398. Bars show the mean and SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. Vehicle; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. MSU; \$$p < 0.05$; \$\$$p < 0.01$; \$\$\$$p < 0.001$ vs. MSC; &$p < 0.05$, &&$p < 0.01$, &&&$p < 0.001$ vs. MSU + MSC. COX-2: cyclooxygenase-2; PG: prostaglandin; PTGES2: prostaglandin E synthase 2; EP: prostaglandin E_2 receptor

in both the MSU and MSU + MSC groups compared with the Control group, and no significant differences between the MSU and MSU + MSC groups.

In summary, MSC administration increased IL-10, IDO-1 and TSG6 expressions in the rabbit SM, without modifying IL-1β or COX-2 levels.

Effect of MSC on the activation of prostaglandin pathway in THP-1-derived macrophages

In view of the early increase in PGE_2 concentration simultaneous with the induction of SPMs and the induction of anti-inflammatory mediators in the tissue mediated by MSC, we decided to delve into the role of PGE_2 in the pro-resolutive response. We employed co-culture experiments with MSCs and THP-1-derived macrophages stimulated with MSU crystals. As shown in Fig. 3A, MSU stimulation increased COX-2 and PTGES in macrophages, simultaneously with an increased expression of EP2, EP3, and EP4 PGE_2 receptors. The addition of MSC significantly super-induced COX-2, PTGES, EP2 and EP3 expressions, in comparison to MSU effect, while EP4 expression decreased. The inhibition of PGE_2 synthesis in MSC cells employing a previous incubation of these cells with NS-398 was able to significantly supress COX-2 and PTGES expression in macrophages (Fig. 3A). These data indicate the key role of PGE_2 derived from MSC in the induction of these enzymes in macrophages.

Regarding the levels of different prostaglandins in the co-culture media, we observed that the addition of MSU crystals to THP-1-derived macrophages was not able to significantly promote prostaglandin synthesis with respect to the control. However, the simultaneous addition of MSCs significantly super-increased the synthesis of PGE_2, PGD_2 and $PGF_2\alpha$ vs. the MSU group (Fig. 3B). The prior incubation of MSC with the COX-2 inhibitor NS-398 completely abolished the release of PGE_2, PGD_2 and $PGF_2\alpha$ to the co-culture media. These data indicated that MSCs are solely responsible for prostaglandin synthesis under co-culture conditions with macrophages (Fig. 3B).

Therefore, MSC presence in the co-culture enhanced COX-2, PTGES, and EP receptor expression in macrophages and was the main source of PGE_2, PGD_2, and $PGF_2\alpha$ production under co-culture conditions.

Role of the COX-2-PGE2 pathway in MSC-mediated anti-inflammatory and pro-resolving mediators synthesis at 24 h

In line with in vivo results, the addition of MSC significantly over-induced the release of the anti-inflammatory cytokine IL-10, and increased LXA_4 and RvD1 concentrations in the co-culture media in comparison to MSU-stimulated macrophages (Fig. 4A). Interestingly, the prior incubation of MSC with a COX-2 inhibitor completely inhibited the release of all these mediators to the culture media, in comparison to MSU + MSC cells. Furthermore, the presence of the EP2 and EP4 receptor antagonists inhibited IL-10 release induced by MSCs. No effect was observed for LXA_4 and RvD1 synthesis, which maintained elevated levels in the culture media (Fig. 4A).

We then tested the regulation of the SPM receptors GPR32 and FPR2, which mediate the pro-resolutive signalling of LXA_4 and RvD1. The addition of MSU as an inflammatory challenge did not alter the gene expression of these receptors in THP-1-derived macrophages. In contrast, the addition of MSCs induced an increase in the gene expression of both receptors in the MSU + MSC group compared to the MSU group (Fig. 4B). This suggests that MSCs not only promote resolutive pathways by stimulating the synthesis of active SPM mediators but also increase the expression of their receptors in macrophages. In summary, MSCs enhanced IL-10, LXA_4, and RvD1 release and increased GPR32 and FPR2 expression in macrophages, while COX-2 inhibition in MSCs suppressed cytokine and SPM production.

Protein expression of lipoxygenases in THP-1 and MSC co-cultures stimulated with MSU

The synthesis of the different SPMs is mediated by different lipoxygenases such as LOX-5, LOX-12 and LOX-15. We studied the protein expression of these enzymes separately in both THP-1-derived macrophages and MSCs, to analyse which cell could be responsible for the release of SPMs to the co-culture media. Regarding THP-1, MSU stimulation decreased LOX-5 expression, while no change was observed for LOX-12 or LOX-15 (Fig. 5A). The co-culture of these cells in an inflammatory milieu (MSU presence) significantly increased the synthesis of LOX-12 in MSCs. A similar trend was observed for LOX-15, in comparison to unstimulated MSCs (Fig. 5A).

Temporal analysis of PGE_2 and LXA_4 induction in MSC and THP-1 co-culture supernatants at short time points

Given that MSC presence within an inflammatory milieu markedly enhanced PGE_2 and SPM release at 24 h (Figs. 3B and 4A), we aimed to determine whether these mediators were released concurrently or exhibited distinct temporal patterns. Therefore, we performed co-culture experiments at different short times (1–6 h). MSU challenge did not modify PGE_2 levels within this period in comparison to vehicle (Fig. 5B). MSC presence induced a significant release of PGE_2 starting at 4 h of co-culture. From that point on, the co-culture of both cell types in an inflammatory situation markedly enhanced PGE_2 release in comparison to that observed in MSU-stimulated macrophages (Fig. 5B). However, under the same culture conditions, LXA_4 release was not significantly enhanced until 6 h of co-culture of both cell types in an inflammatory setting (Fig. 5B). This data suggest that MSC-mediated PGE_2 induction would precede LXA_4 release in co-culture with THP-1 macrophages.

Effect of COX-2 Inhibition or FPR2 Blockade on THP-1 phagocytosis and M2-polarisation

Next, we sought to determine whether the release of pro-resolving mediators could modulate the efferocytic capacity, macrophage polarization, and the release of different mediators in co-culture. We also tested whether these functional capacities were mediated by PGE_2 release, or FPR2 activation. For these purposes we employed M1 polarized THP-1 derived macrophages. MSC presence induced a significant increase in the phagocytosis of *E.coli* fluorescent particles by MSU-activated macrophages, in comparison to MSU alone (Fig. 6A). This result was further confirmed by confocal imaging, showing a marked increase in the fluorescent signal in the pHrodo + MSC group compared to the pHrodo group. (Fig. 6B). Interestingly, the addition of the COX-2 inhibitor significantly decreased the phagocytosis ability induced by MSCs. Besides, the presence of the FPR2 antagonist abrogated the MSC-mediated prophagocytic effect (Fig. 6A).

We also characterized the expression levels of efferocytic markers in THP-1-derived macrophages in these experiments. MSU crystals evoked a drop in the expression of CD68, CD36 and MerTK in THP-1 in comparison to vehicle (Fig. 6C). However, MSC incorporation significantly increased the expression levels of CD68, FPR2 and MerTK in these cells compared to MSU (Fig. 6C). This effect was abrogated for all these markers in the presence of a COX-2 inhibitor (Fig. 6C). Furthermore, the addition of the FPR2 antagonist in the presence of MSC significantly decreased CD68, MerTK and FPR2 gene expression in comparison to MSU + MSC (Fig. 6C). In these experiments, MerTK presence was also analyzed by confocal imaging. A notable increase in the fluorescent signal for MerTK was observed in MSU + MSC group vs. MSU group. A phenotypic change was also observed in macrophages, from roundish cell bodies in MSU group to elongated cell bodies in MSU + MSC (Fig. 6D).

Concerning the expression of macrophage polarization markers, MSU crystal stimulation significantly increased the pro-inflammatory M1 marker CD80, while a decrease in the anti-inflammatory M2 markers CD206 and CD163 was observed (Fig. 6E). In contrast, MSCs addition decreased CD80 and significantly increased CD206, CD163 and ARG1, compared to the effect of MSU (Fig. 6E). COX-2 inhibition exclusively in MSC blocked the effect observed for the polarization markers CD80 and CD163 compared to the MSU + MSC group (Fig. 6E). In addition, the FPR2 antagonist significantly decreased CD163 gene expression without altering any other markers, in comparison to MSU + MSC group (Fig. 6E).

To confirm the gene expression results of M2 polarization markers at the protein level, we analysed ARG1 protein expression. Consistent with the gene expression data, ARG1 levels were significantly decreased in M1 macrophages stimulated with MSU crystals (Fig. 6F). The addition of MSCs to the co-culture significantly reversed this effect, partially restoring ARG1 expression. In contrast, co-culture with MSCs pretreated with the COX-2 inhibitor abolished this increase, whereas FPR2 antagonism did not produce any significant effect compared with MSC-treated macrophages (Fig. 6F).

To further assess the functional impact of MSCs on the inflammatory and pro-resolving response of macrophages, we measured IL-1β and RvD1 levels in the culture supernatants. Stimulation of M1 polarized-THP-1–derived macrophages with MSU crystals significantly increased IL-1β release compared with control cells, whereas RvD1 levels remained low (Fig. 7). Co-culture with MSCs significantly reduced IL-1β release and increased RvD1 production. IL-1β release employing MSCs pretreated with the COX-2 inhibitor was not different to that measured in MSU-stimulated macrophages, while RvD1 induction was completely prevented. Blockade of FPR2 did not significantly affect the release of either mediator compared with untreated MSCs (Fig. 7).

In summary, MSC presence in the co-culture enhanced macrophage phagocytic capacity, increased the expression of efferocytic and M2 polarization markers, reduced M1 marker expression, and shifted the balance of soluble mediators by suppressing IL-1β release and inducing RvD1 production. These effects were abolished by COX-2 inhibition in MSCs, while some of these effects were partially reversed by FPR2 blockade.

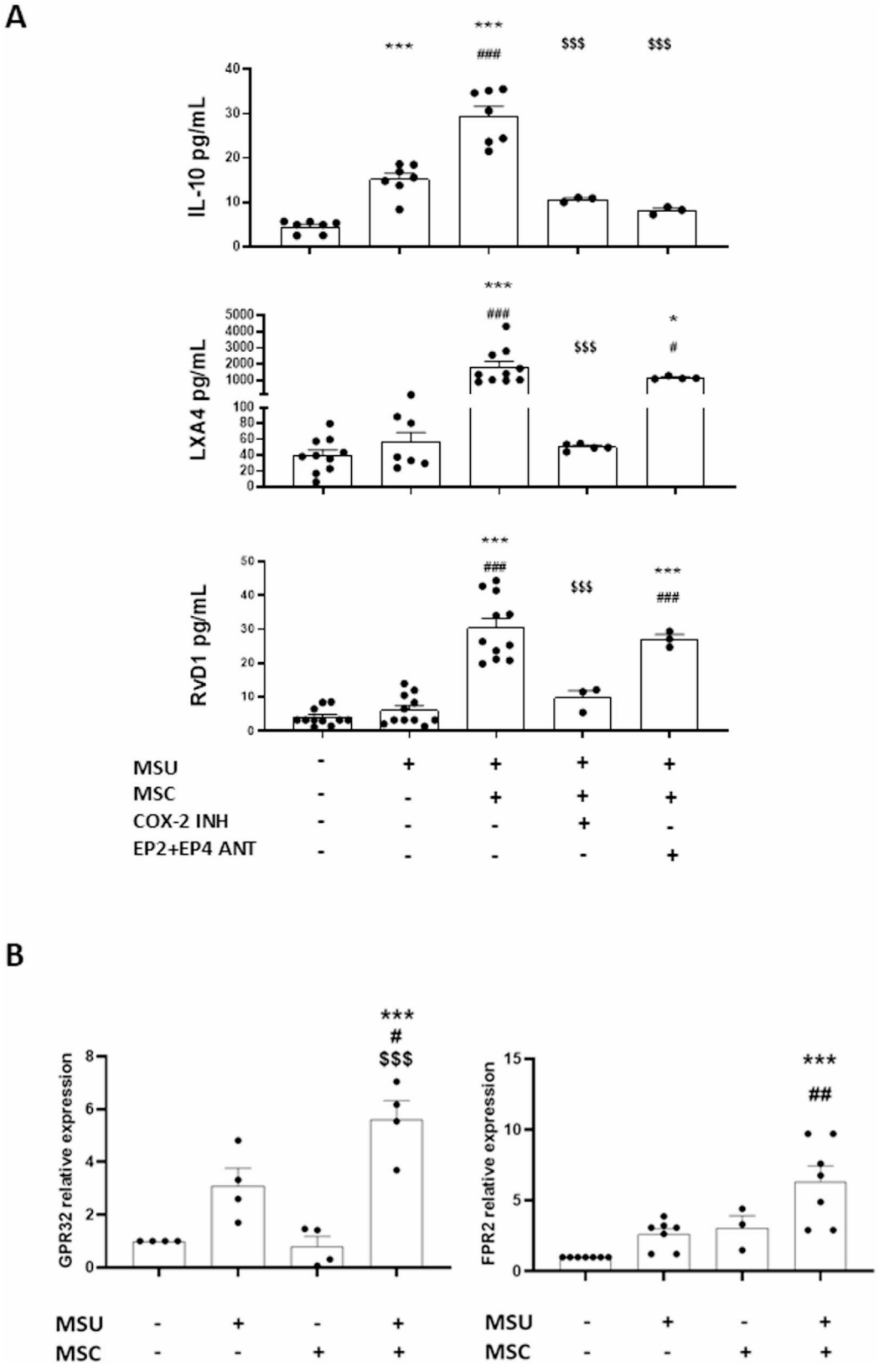

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Fig. 4 MSC addition induced anti-inflammatory and pro-resolving responses in a COX-2-dependent manner. **A** Supernatant IL-10, LXA_4 and RvD1 levels from MSC-THP-1 cocultures in the presence or absence of the COX-2 inhibitor NS-398 or EP2 plus EP4 antagonists. **B** Gene expressions of SPM receptors GPR32 and FPR2 in THP-1 derived macrophages in the presence or absence of MSCs in the co-culture. Bars show the mean and SEM. *$p < 0.05$; ***$p < 0.001$ vs. Vehicle; #$p < 0.05$; ###$p < 0.001$ vs. MSU; $$$$p < 0.001$ vs. MSU + MSC. ANT: antagonist; COX-2: cyclooxygenase-2; EP: prostaglandin E_2 receptor; IL: interleukin; INH: inhibitor; LXA_4: Lipoxin A4; MSC: mesenchymal stem cell; MSU: monosodium urate; Rv: resolvin

Discussion

The resolution of acute inflammation is an active process driven by the inflammatory response itself and the sequential biosynthesis of SPMs, within a complex and not yet fully deciphered scenario. These mediators promote active resolution, limiting excessive immune activation, and facilitating tissue repair and restoring homeostasis. Our study provides a detailed analysis of the temporal dynamics and profile of SPMs and selected precursors in the synovium during acute joint injury, and their temporal modulation by MSC therapy. These cells shortened both the duration and the severity of this self-limiting condition [11]. This effect coincided with a pronounced local induction of PGE_2 biosynthesis and increased expression of two key receptors in the resolution process, as EP4 and FPR2. We observed that DHA and 14-HDHA and active products such as LXA_4, were markedly decreased in the SM 24 h after tissue injury. Some remained reduced even after 72 h, in comparison with healthy tissue. Previous data in a self-limiting serum-transfer arthritis model also showed reduced SPMs in the inflamed whole paw, but spontaneous resolution was characterized by local increases in some of these mediators [34]. Consistent with our results, SPM precursors such as 12-HETE, 15-HETE, 17-HDHA, 14(S)-HDHA, 12-HEPE and 15-HEPE were downregulated in the serum of gouty patients compared to hyperuricemic and normouricemic individuals [35]. In addition, a negative correlation between IL-1β and LXA_4 serum levels of patients with acute gout has been reported [36]. All these data lead to the hypothesis that acute joint inflammation generates extensive dysregulation of the pro-resolving system both locally and systemically, as described for acute inflammation associated with myocardial infarction [37].

The divergent kinetics observed for MaR1, PD1, and PDX in our model highlight the complexity of SPM time and concentration regulation during resolution. While MaR1 and PD1 showed an early rise at 24 h followed by a return to baseline by 72 h, PDX levels continued to increase at the later time point. This transient pattern is in line with the classification of SPMs as autacoids —short-lived, locally acting mediators whose activity is tightly regulated by metabolic inactivation and clearance pathways [38, 39]. Even when precursor pools such as DHA or 14-HDHA remain available, enzymatic inactivation (e.g., by 15-prostaglandin dehydrogenase or CYP450-mediated ω-oxidation) [39–41] may account for the disappearance of certain SPMs. Some metabolites retain pro-resolving bioactivity (e.g., 20-OH-RvE1, 22-OH-PD1, 22-OH-MaR1) [39, 42, 43], whereas others lose activity completely [39, 41]. The persistence of PDX, in contrast, suggests that distinct SPM species may be differentially regulated and contribute at different stages of the resolution response. Altogether, these findings support the view that SPM kinetics are highly complex and incompletely understood, with individual mediators playing temporally distinct roles in orchestrating the resolution of inflammation.

The effects of MSCs in acute sterile inflammation are mainly linked to their capacity to regulate the activation of the myeloid compartment. In particular, MSCs promote macrophage polarization toward a pro-resolving and anti-inflammatory phenotype [11, 44, 45]. There is limited evidence on their ability to produce SPMs in vivo thereby accelerating the resolution of inflammation. Human MSC have the enzyme machinery, 5-,12- and 15-LOX, for SPM biosynthesis [46–48] and are rich in SPM precursors such as AA or DHA [49, 50]. In mice with LPS-induced acute lung injury, MSCs improved survival and limited lung damage, partly through LXA_4 synthesis. This indicates that other mechanisms may further contribute to their therapeutic effects [51]. In vitro, human periodontal stem cells can produce and release D- and E-series Rvs, LXs and Mars [52]. Supplementation with DHA further enhanced the pro-resolving capacity of cultured MSC through increased SPM production [53, 54]. In our study, systemic MSC administration after an acute gout flare rapidly increased DHA and 14-HDHA levels in the inflamed tissue, providing substrates for the synthesis of downstream active products. MSC treatment also induced the local production of Mar-1 and – 2, PD-1 and PDX within 24 and 72 h, RvD4 at 72 h, and LXA_4 at 24 h. This study is pioneering in demonstrating that systemic MSC administration in a model fully reproducing acute gout [27, 55] acts as a broad-spectrum replacement therapy for SPMs, effectively enhancing their local levels in the affected tissue. These data are consistent with findings in sepsis models, where silencing 5-LOX and 12/15-LOX in MSCs abolished their beneficial effects [56]. Together with previous findings in this model, our data show that synovial SPM replenishment at 24 h was associated with a shorter and milder inflammatory flare, followed by a decrease in synovial histological damage and increased M2 macrophage polarization at 72 h after MSU injection [11]. Interestingly, MSC treatment also

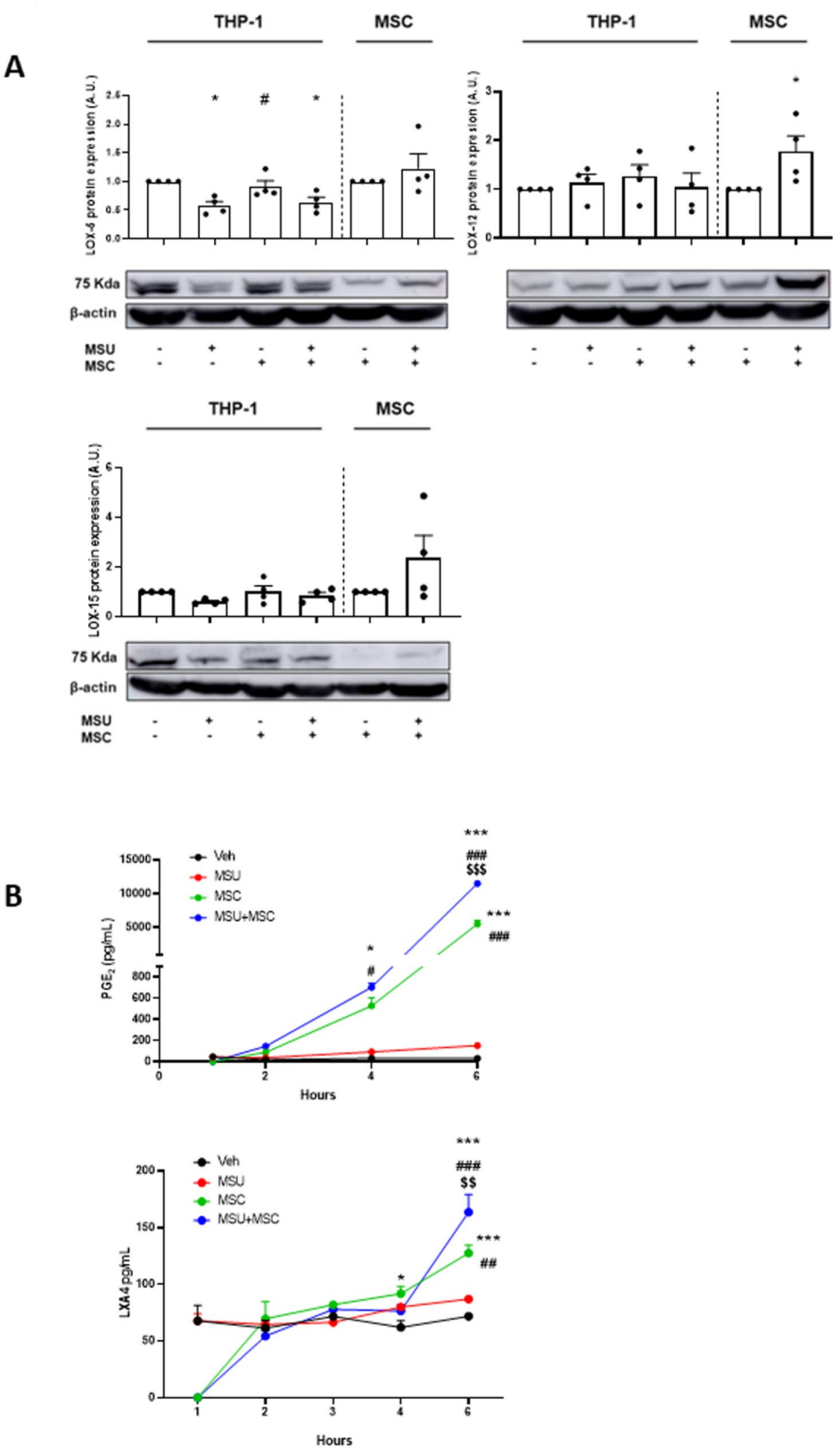

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Fig. 5 **A** Protein expressions of LOX – 5, LOX-12 and LOX-15 in THP-1 derived macrophages or MSCs in co-culture after 24 h of incubation. Cells were co-cultured in the conditions described, and then cell extracts were separately recovered. Bars show the mean and SEM. *$p < 0.05$ vs. Vehicle; #$p < 0.05$ vs. MSU. **B** PGE_2 and LXA_4 concentrations in the THP-1-derived macrophages and MSCs co-culture media at different hours of co-culture. Each data point represents the mean and SEM at each time point. *$p < 0.05$; ***$p < 0.001$ vs. Vehicle at each time point; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. MSU at each time point; $$$p < 0.01$; $$$$p < 0.001$ vs. MSC at each time point. PG, prostaglandin; LXA_4: Lipoxin A4; MSC: mesenchymal stem cell; MSU: monosodium urate

increased anti-inflammatory mediators such as IL-10, IDO-1 and TSG6 in the SM at 24 h, which have been previously linked to MSC therapeutic effects [29, 57]. In contrast, IL-1β or COX-2 expressions were unchanged at this early time. Therefore, MSC-induced SPMs and anti-inflammatory markers preceded the downregulation of pro-inflammatory factors, which became evident at 72 h [11]. Additionally, SPM increases coincided with a synovial decrease in ALOX-5 and ALOX-12 gene expression, while ALOX-15 remained unchanged, suggesting feedback regulation of LOX enzymes [58, 59]. MSCs also increased FPR2 expression, potentially enhancing the action of LXA_4 and other SPMs, as also reported in diabetic kidney models treated with MSCs [60].

It seems reasonable to assume that the early increase in SPM levels underlies the pro-resolutive effect of MSC treatment in gouty rabbits. Consistent with this exogenous administration of LXA_4, PDX, RvD4, or RvD1 decreased joint swelling, synovitis, or the local production of inflammatory mediators in other experimental models [34, 61–63]. Furthermore, SPMs, including RvE1 and Mar-1, synergized with MSCs to enhance their in vitro immunomodulatory capacity, increasing IL-10 and TGF-β production [64].

In MSC-treated animals, the greatest increase in local SPM concentration at 24 h coincided with the peak of PGE_2 concentration. In contrast, the MSU group showed lower synovial PGE_2 at 24 h, which continued to rise until 72 h, consistent with delayed resolution. Moreover, MSC treatment upregulated COX-2 and EP4 receptor expression, supporting an anti-inflammatory role of PGE_2 [10, 65]. Previous data indicated that PGE_2 increase preceded M2 macrophage polarization and NLRP3 inflammasome inhibition in MSC-treated animals [11]. In line with these results, our in vitro experiments demonstrated that MSC addition to MSU-stimulated macrophages increased COX-2 and PGES gene expression, as well as the concentration of PGE_2, PGD_2 or $PGF_2\alpha$s. Moreover, we found increased EP2 receptor levels, which complements EP4 in cAMP activation and contribute to the anti-inflammatory effects of PGE_2 [65, 66]. Importantly, selective COX-2 inhibition restricted to MSCs abolished the induction of these enzymes and lipid mediators, indicating that MSCs are the primary source of prostaglandins in our system.

MSCs significantly enhanced the release of the anti-inflammatory mediator IL-10 and the SPMs LXA_4 and RvD1 to the co-culture medium, in agreement with in vivo results. In addition, they also upregulated the gene expression of the SPM receptors GPR32 and FPR2 in MSU-stimulated macrophages, favoring a pro-resolving phenotype [67, 68]. Interestingly, COX-2 inhibition in MSCs completely abrogated the release of both anti-inflammatory and pro-resolving mediators, confirming that prostaglandin synthesis by MSCs is essential to trigger resolution. In our functional assays we assessed macrophage efferocytic capacity and the expression of efferocytosis-related markers such as MerTK, CD68 and FPR2—all associated with the pro-resolving macrophage phenotype [67, 69, 70]. These assays provide functional evidence of a pro-resolving response to MSCs, extending our understanding of macrophage plasticity beyond conventional surface markers. These findings are in line with our previous in vivo observations showing that MSC administration induces macrophage repolarization at 72 h in the SM of MSU-MSC rabbits, as reflected by increased ARG-1/RAM11 and CD163/RAM11 ratios [11].

In our in vitro experiments, when COX-2 was inhibited only in MSCs, we observed a marked reduction in both efferocytosis and the expression of efferocytic markers in MSU-stimulated macrophages. This effect was attributed to impaired SPM release, as confirmed by the decreased production of RvD1 and LXA_4 following MSC-specific COX-2 inhibition. Furthermore, blocking FPR2 nearly abolished the increase in efferocytic capacity and the expression of pro-resolving markers in macrophages. Altogether, these findings suggest that prostaglandins synthesized by MSCs play a pivotal role in driving macrophage pro-resolving functionality via SPM production. Interestingly, unlike COX-2 inhibition, the increase in LXA_4 and RvD1 concentration in the co-culture media was not impaired by EP2/EP4 antagonists. These data suggest that additional prostanoids, acting through alternative receptors, may also contribute to the MSC-induced pro-resolving response. Indeed, increased levels of $PGF_2\alpha$ and PGD_2— via DP1 receptor activation—have been associated with resolution of inflammation in models of acute intestinal and pulmonary inflammation [71, 72].

To confirm that prostaglandin release is an early event triggered by MSCs in an inflammatory environment, we measured PGE_2 and LXA_4 concentrations in the co-culture supernatants at early time points. PGE_2 levels rose

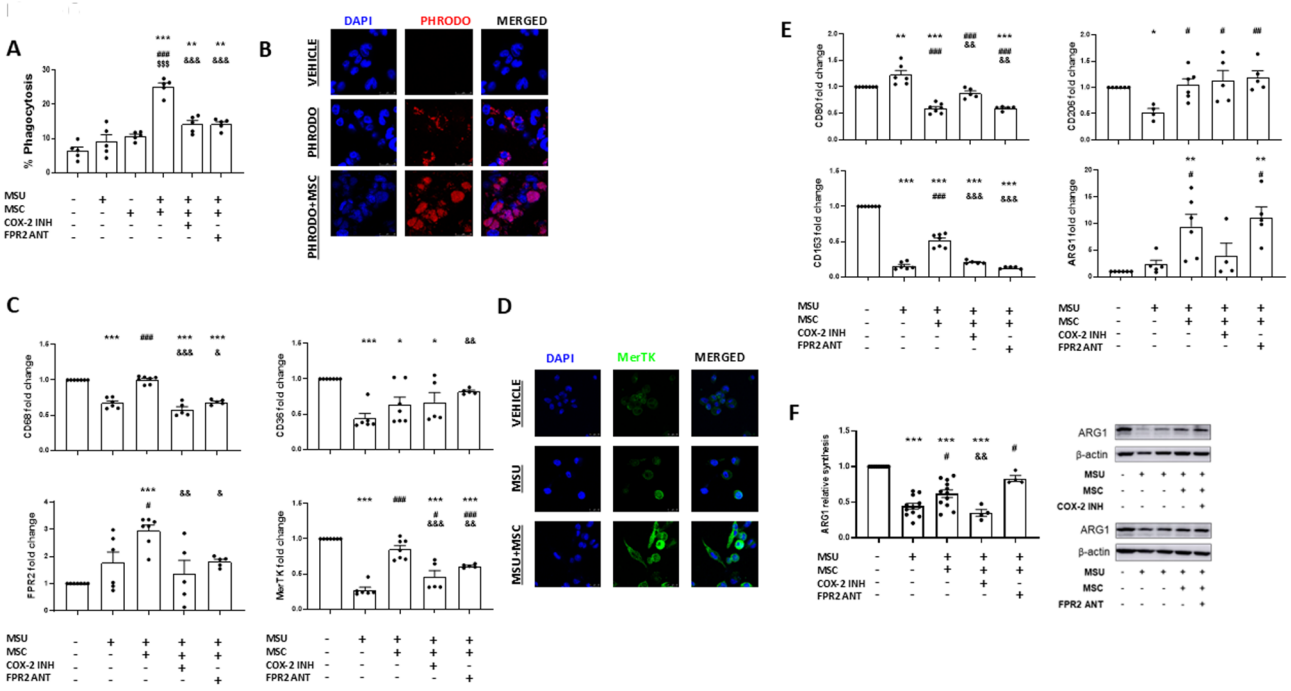

Fig. 6 **A** Percentage of *E. coli* fluorescent Phrodo particles phagocyted by MSU-stimulated M1 polarized THP-1-derived macrophages after 3 h of culture in the presence or absence of MSCs, and/or the COX-2 inhibitor NS-398, and/or the FPR2 antagonist WRW4. **B** Confocal microscopy images of a representative phagocytosis experiment. **C** Gene expression of pro-efferocytic markers in M1 polarized THP-1 derived macrophages in the presence or absence of MSCs, and/or the COX-2 inhibitor NS-398, and/or the FPR2 antagonist WRW4. **D** Confocal microscopy images of a representative MerTK immunohistochemistry experiment. **E** Gene expression of M1 and M2 polarization markers in M1 polarized THP-1-derived macrophages in the presence or absence of MSCs, and/or the COX-2 inhibitor NS-398, and/or the FPR2 antagonist WRW4. **F** Western-blot studies of ARG1 protein synthesis in M1 polarized THP-1-derived macrophages in the presence or absence of MSCs, and/or the COX-2 inhibitor NS-398, and/or the FPR2 antagonist WRW4. A representative western-blot is shown. Bars show the mean and SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. Vehicle; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. MSU; $$p < 0.05$; $$$p < 0.01$; $$$$p < 0.001$ vs. MSC; &$p < 0.05$, &&$p < 0.01$, &&&$p < 0.001$ vs. MSU + MSC. ANT, antagonist; ARG, Arginase; COX-2: cyclooxygenase-2; FPR2: formyl peptide receptor 2; INH, inhibitor; MerTK, Mer Tyrosine Kinase

significantly before the appearance of LXA_4, supporting the hypothesis that an early increase in PGE_2 acts as an upstream signal in MSC-mediated responses, initiating the synthesis of SPMs. These mediators can then act on macrophages, enhancing their efferocytic capacity and promoting macrophage polarization toward an anti-inflammatory, pro-resolving, tissue-repairing phenotype.

SPMs biosynthesis in acute inflammation is largely a transcellular process involving cooperation among different cell types —mainly macrophages, neutrophils, and platelets [73, 74]. However, in vitro studies have shown that certain isolated cell types can also synthesize specific SPMs. For instance, M1 macrophages may lack the capacity to produce biologically relevant amounts [73], whereas limited evidence suggests that MSCs can intrinsically synthesize mediators, such as Rv, Mars or Ps [52]. In our study, THP-1-derived macrophages synthesized 5-LOX, 12-LOX, and 15-LOX under inflammatory conditions, but their levels remained unchanged in the presence of MSCs. In contrast, in MSCs co-cultured with MSU-stimulated macrophages, 12-LOX protein expression was significantly upregulated, with a similar trend for 15-LOX. These findings suggest that, under our experimental conditions, LOX enzymes responsible for SPM biosynthesis are primarily expressed by MSCs. Overall, our data indicate that MSCs and macrophages cooperate to mount an anti-inflammatory and pro-resolving response. MSCs appear to be the main source of PGE_2, and although both cell types can release anti-inflammatory mediators, SPM synthesis may rely predominantly on MSCs.

While our study integrates both in vitro and in vivo approaches, certain limitations should be acknowledged. Our in vivo study confirmed early molecular changes following MSC administration, including increased synovial PGE_2 and SPMs; however, the precise cellular sources of these mediators could not be definitively determined. It is plausible that SPMs arise through transcellular biosynthesis involving enzyme and substrate sharing among multiple cell types [73–75]. In acute synovitis enriched in neutrophils and platelets, LXA_4 production can occur through 5-LOX/12-LOX cooperation, while macrophages are major sources of Mars and RvDs during resolution. Additionally, synovial fibroblasts express 15-LOX

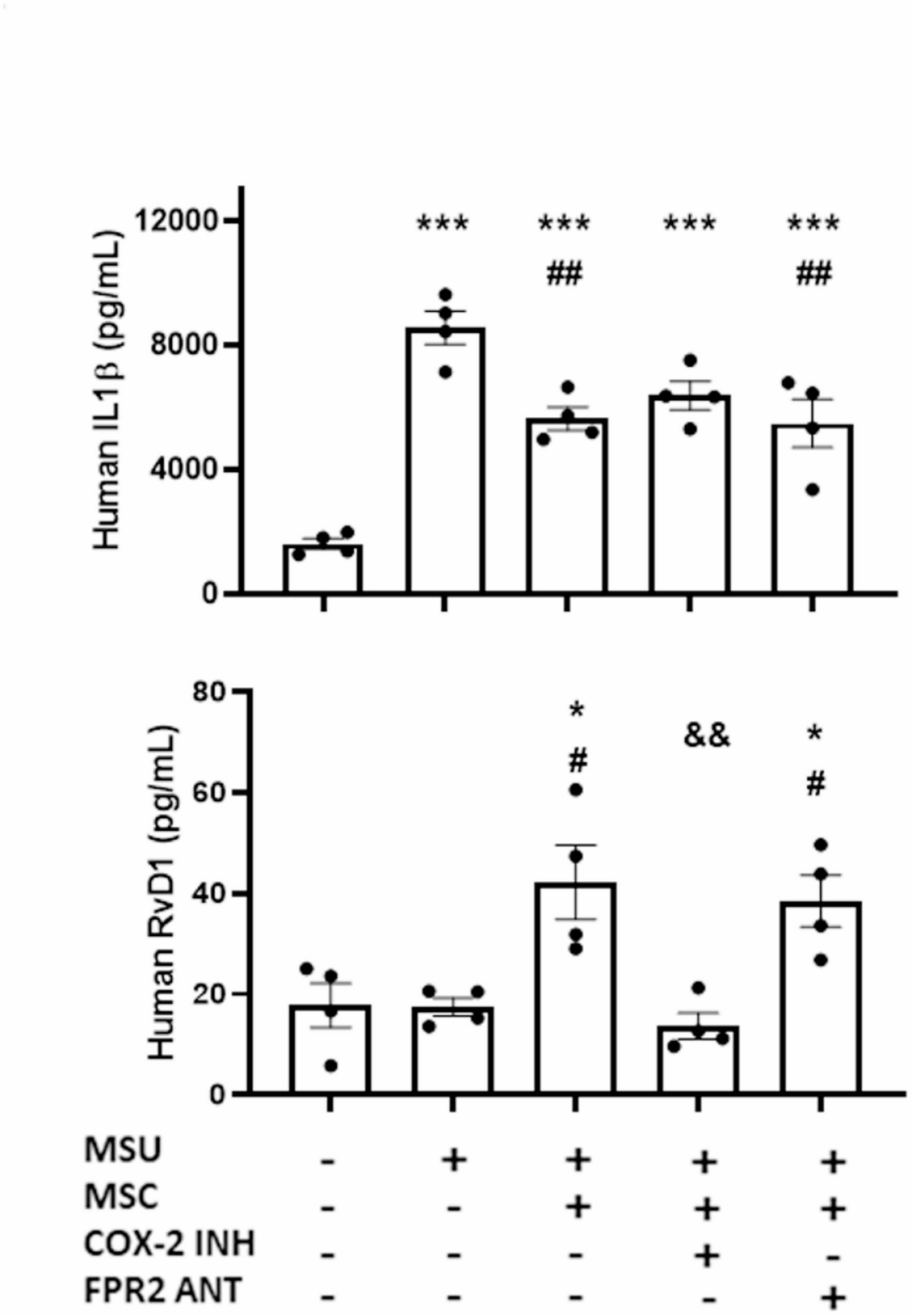

Fig. 7 MSC addition induced anti-inflammatory and pro-resolving responses in a COX-2-dependent manner in M1 polarized-THP-1 derived macrophages. Supernatant IL-1β and RvD1 levels from MSC-THP-1 cocultures in the presence or absence of the COX-2 inhibitor NS-398 or the FPR2 antagonist. Bars show the mean and SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. Vehicle; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. MSU; &$p < 0.05$, &&$p < 0.01$, &&&$p < 0.001$ vs. MSU + MSC

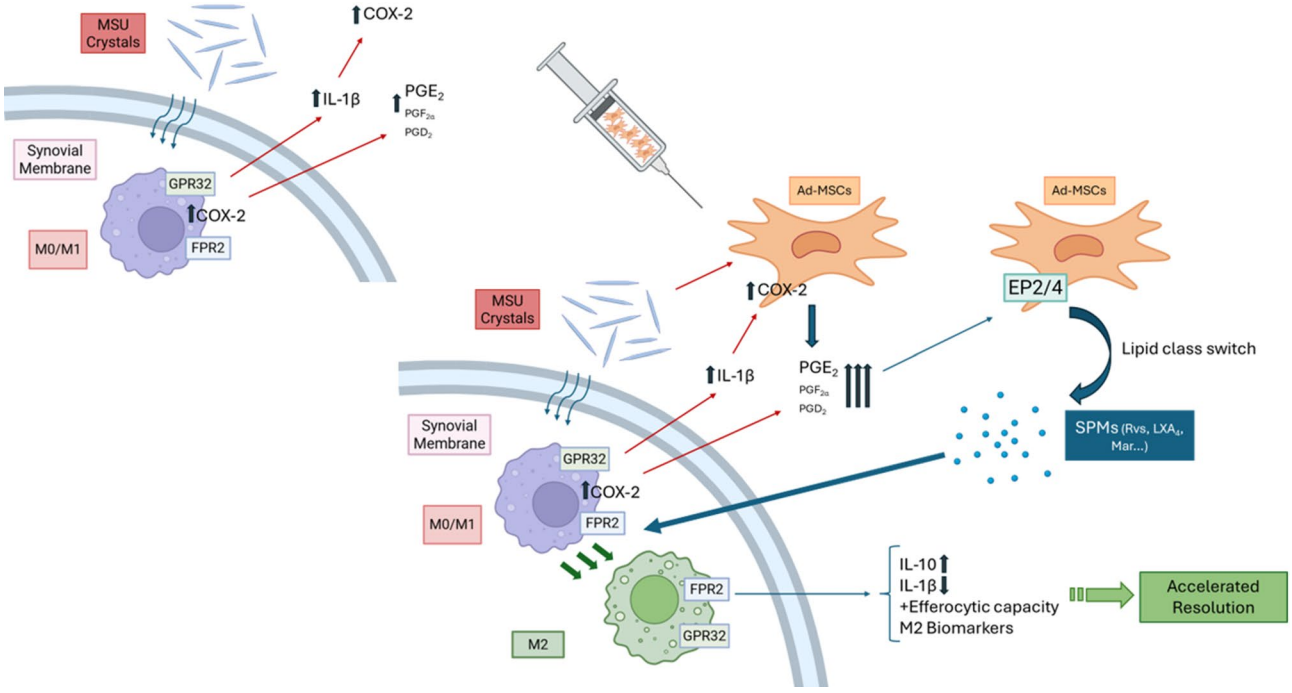

Fig. 8 Schematic representation of the proposed mechanism. Upper left panel, according to our data, the deposition of MSU crystals in the joint space triggers an inflammatory response in synovial cells, particularly in resident macrophages (red arrows), leading to increased release of pro-inflammatory cytokines (e.g., IL-1β) and lipid mediators (e.g., PGE_2) through COX-2 induction. Bottom right panel, the injection of MSCs in this pro-inflammatory environment further amplifies PGE_2 production via COX-2 activation, along with the release of other lipid mediators. This PGE_2 super-induction promotes a lipid class switch (blue arrows) driven by lipoxygenase activation, resulting in increased local levels of both precursors and active specialized pro-resolving mediators (SPMs), as well as anti-inflammatory cytokines such as IL-10. This pro-resolving environment induces a polarization shift in synovial macrophages toward a phenotype with enhanced efferocytic capacity, expression of M2 markers, and the ability to produce SPMs and additional anti-inflammatory mediators, ultimately accelerating the resolution of the acute inflammatory flare. Our data show that selective COX-2 inhibition in MSCs blocks this pro-resolving response, likely by preventing PGE_2 and related lipid super-induction. In addition, some of these pro-resolving effects are mediated through the SPM receptor FPR2, particularly the induction of IL-10 release

isoforms, which may locally supply SPM precursors [76]. Although MSCs are capable of producing SPMs under specific conditions, our current experiments were not designed to distinguish MSC-derived from host-derived SPMs. Future studies combining cell-type–resolved lipidomics, stable-isotope tracing, spatial approaches, and conditional enzyme deletion will be essential to delineate the cellular contributors in vivo. Another limitation is that our lipidomic analysis focused on a limited number of mediators, likely underestimating the complexity of the lipid signaling network.

In addition, we used THP-1-derived macrophages as a standardized and reproducible model to dissect MSC–macrophage interactions. Although these cells cannot fully capture the complexity of primary human macrophages, they provide a robust platform for mechanistic analyses, particularly for processes such as polarization and its functional implications [77, 78]. Notably, the responses observed in our in vitro system—early COX-2/PGE_2 induction, subsequent SPM generation, and macrophage polarization—closely paralleled those detected in vivo in rabbit synovium. Moreover, in a previous work we confirmed that MSCs similarly induced a super-induction of COX-2/PGE_2 in PBMC-derived macrophages from healthy individuals, further supporting the translational relevance of this model [11]. Future studies with primary human macrophages and synovial explants will be required to validate and extend these observations in clinically relevant contexts.

Finally, species-related considerations should also be taken into account. Rabbits differ from humans in urate metabolism, since they express uricase and efficiently convert urate to allantoin, which may render MSU-driven flares more self-limiting than in patients. Moreover, interspecies differences in receptor pharmacology relevant to resolution (e.g., ALX/FPR2 and EP2/EP4 signaling, the human-specific GPR32) may affect the magnitude and timing of the PGE_2–SPM axis. Another important limitation is the xenogeneic administration of human MSCs in immunocompetent rabbits. Although MSCs are often described as immune-evasive, they are not fully immune-privileged, and their survival, biodistribution, and paracrine activity may be modulated by species mismatch. Taken together, these factors indicate that our results should be interpreted as proof-of-concept evidence for an MSC-driven pro-resolving program in

acute gout rather than a direct reproduction of the clinical scenario.

Conclusion

In summary, MSCs exposed to an inflammatory environment synthesize a variety of mediators, with both pro- and anti-inflammatory properties. A marked induction of COX-2 appears to lead local PGE_2accumulation, which may serve as an upstream signal favoring a lipid mediator class switch. Through EP2/4 receptor signaling, this early PGE_2 increase may halt pro-inflammatory lipid synthesis while initiating the production of pro-resolving lipid mediators, likely within MSCs themselves (Fig. 8). In parallel, the expression and release of additional anti-inflammatory mediators are promoted in both MSCs and surrounding immune cells such as macrophages. This pro-resolving secretome enhances macrophage efferocytosis and promotes their polarization towards an anti-inflammatory, pro-resolving phenotype. In particular, SPMs acting through the FPR2 receptor would contribute to this functional polarization of macrophages. This integrated mechanism is summarized in Fig. 8, which provides a visual overview of the proposed pathway underlying the pro-resolving actions of MSCs.

Abbreviations

AA	Arachidonic acid
ARG1	Arginase 1
COX-2	Cyclooxygenase-2
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EP	Prostaglandin E2 receptor
EPA	Eicosapentaenoic acid
FPR2	Formyl peptide receptor 2
GPR32	G protein-coupled receptor 32
H-DHA	Hydroxy docosahexaenoic acid
HEPE	18-hydroxyeicosapentaenoic acid
IDO-1	Indoleamine 2,3-dioxygenase-1
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LOX	Lipoxygenase
LT	Leukotriene
LX	Lipoxin
Mar	Maresin
MerTK	Mer tyrosine kinase
MSCs	Mesenchymal stem cells
MSU	Monosodium urate
NF	Nuclear factor
NLRP-3	NOD-like receptor family pyrin domain containing-3
P	Protectin
PG	Prostaglandin
PMA	Phorbol 12-myristate 13-acetate
PTGES	Prostaglandin E synthase
PUFAs	Polyunsaturated fatty acids
Rv	Resolvin
SM	Synovial membrane
SPE	Solid-phase extraction
SPMs	Specialized pro-resolving mediators
TSG6	Tumour necrosis factor-stimulated gene 6
TX	Thromboxane

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

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Author contributions

IB-A and RL were in charge with conception, design, analysis and interpretation of data. IB-A, CV, A I-M, and IQ-R performed animal and cellular experiments and contributed to the acquisition of data. IB-A, GH-B, AM and RL were involved in drafting the article and/or revising it critically for important intellectual content. All authors have read and approved the definitive version to be published.

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Data availability

Corresponding author (RL) has full access to overall data and takes responsibility for the integrity and accuracy of the data analysis. All data supporting the findings of this study are available as supplementary material.

Declarations

Ethics approval and consent to participate

All methods were performed in accordance with the relevant guidelines and regulations. This study involves the use of MSCs obtained from the adipose tissue from human healthy donor lipoaspirates after informed consent according to the approval by the Ethics Committee of Hospital Fundación Jiménez Díaz (Project "mRNA engineered mesenchymal stromal cells: a new generation of cell therapy for inflammation", approved on October 19, 2017, PIC09/2016_Am). All animal experiments were performed by procedures approved by the Institutional Ethics and Animal Welfare Committee of IIS-FJD (Project "Mesenchymal stromal cells as inducers of inflammation resolution-specific mediators in the treatment of gouty arthritis. PROEX 139.7/20, approved on June 19, 2020).

Consent for publication

Not applicable.

Competing interests

RL, IB-A and GH-B have filed a patent application (#23382935.7) on the use of SPMs mixtures for the treatment of diseases or conditions that involve acute inflammation. The authors declare no others conflict of interest.

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