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Macrophage-induced reactive oxygen species promote myometrial contraction and labor-associated mechanisms

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Running title: Macrophage-induced oxidative stress in labor

Summary sentence: Myometrial cell contraction and differentiation require prior activation of macrophages and subsequent release of reactive oxygen species.

Keywords: Oxidative stress, Differentiation, Labor, Macrophage, Myocyte, Cell culture

Abstract

At labor, the myometrium is infiltrated by a massive influx of macrophages that secrete high levels of pro-inflammatory cytokines inducing the expression of specific labor-associated markers. However,

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the interactions between myocytes and macrophages and the role of macrophages in the myometrium at labor remain to be elucidated. In this work, we studied the role of myometrium-infiltrated macrophages and their interaction with myocytes in lipopolysaccharide-induced preterm labor. A co-culture model of human primary myometrial cells and macrophages was developed and validated. Collagen lattices were used to evaluate myocyte contraction. Differentiation steps were assessed by i) phalloidin and vinculin staining for cytoskeleton reorganization, ii) gap junction protein alpha 1 expression and scrape loading/dye transfer with Lucifer Yellow for gap junction intercellular communication, and iii) calcium imaging for cell excitability. We demonstrated that macrophages favored lipopolysaccharide-induced contraction and early differentiation of myometrial cells. Transwell assays showed that previous activation of macrophages by lipopolysaccharide was essential for this differentiation and that macrophage/myocyte interactions involved macrophage release of reactive oxygen species (ROS). The effects of macrophage-released ROS in myometrial cell transactivation were mimicked by H_2O_2 , suggesting that superoxide anion is a major intermediate messenger in macrophage/myocyte crosstalk during labor. These novel findings provide the foundation for innovative approaches to managing preterm labor, specifically the use of antioxidants to inhibit the initial stages of labor before the contractile phenotype has been acquired. In addition, the co-culture model developed by our team could be used in future research to decipher pathophysiological signaling pathways or screen/develop new tocolytics.

Introduction

Preterm labor (PTL), defined as labor before 37 weeks, remains a major obstetric challenge. It is estimated to affect 11% of pregnancies worldwide, with approximately 15 million babies born prematurely each year [1]. Preterm birth is a major cause of perinatal morbidity and mortality. Indeed, it accounts for 75% of perinatal mortality and more than half of long-term morbidity [2]. The preterm birth rate has risen in most industrialized countries, despite advancing knowledge of risk factors and mechanisms related to PTL, and the introduction of many public health and medical interventions [2].

It is now well established that labor, both preterm and term, is an inflammatory event [3,4] that results from the priming of circulating leukocytes [5,6] and the massive infiltration of macrophages and

neutrophils into the myometrium, which secrete pro-inflammatory cytokines [7–9]. However, it remains unclear whether the inflammatory infiltration of the myometrium is a cause or consequence of labor [10]. Although the interactions between myometrial cells and macrophages remain to be elucidated, macrophages seem to be mainly involved in the onset of labor [7–9]. In lipopolysaccharide (LPS)-treated mice, for instance, macrophage depletion prevented preterm delivery [11] whereas neutrophil depletion had no effect [12]. Our team has also provided some answers about the role of macrophages in labor by highlighting that macrophages were required for the activation of myometrial cells [13]. More precisely, we demonstrated that human preterm laboring myometrium secreted high levels of reactive oxygen species (ROS), with 90% of the production observed in invaded macrophages. Furthermore, macrophage-released ROS induced an increase in the expression of specific labor-associated markers (matrix metalloproteinase 2 [MMP2], prostaglandin-endoperoxide synthase 2 [PTGS2], and cleaved caspase 3) [13]. Uterine labor involves a phenotypic transition including a differentiation stage that consists of an increased expression of a cassette of genes that encode proteins such as ion channels, agonist receptors and gap junctions (GJ) [14]. More precisely, the early differentiation stage includes three steps: i) a reorganization of the cytoskeleton of myometrial cells, ii) an increase in the expression of gap junction protein alpha 1 (GJA1), allowing functional GJ formation, and iii) an increase in the Na⁺ and Ca²⁺ ion channels that control myometrial cell excitability [14]. Once differentiated, myometrial cells can optimally respond to the increased production of contractile agonists (e.g. oxytocin, stimulatory prostaglandins) that in turn drive the contractions required for the delivery of the fetus. Current tocolytic therapy (e.g. calcium channel blockers) inhibits myometrial contractions. However, the efficacy of such treatment in terms of pregnancy delay and neonatal improvement has proven limited [15]. We believe that the paradigm of PTL management has evolved and that new therapeutic agents should target the initial stages of labor, such as the differentiation phase. Moreover, given the increasingly recognized role of macrophage-induced oxidative stress in labor-associated mechanisms [13,16,17], we should consider antioxidants as potential tocolytic agents.

Based on these data, we wished to explore the role of macrophages in the contraction and early differentiation processes of myometrial cells in order to explain the mechanism behind macrophage infiltration in labor onset.

Materials and Methods

- *Drugs and equipment*

LPS from *Escherichia coli* (serotype 055:B5; L2880), H₂O₂ (H1009), glutathione (GSH; G6013), catalase (C1345), phorbol 12-myristate 13-acetate (PMA; P8139), anti-vinculin antibody (clone hVIN-1; V9131), Lucifer Yellow (LY; L0259) solution and Transwell inserts with 0.4µm pore diameter were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Granulocyte macrophage-colony stimulating factor (GM-CSF; 130-095-372) was obtained from Miltenyi Biotec (Paris, France). Rhodamine dextran (RD, D1824, M_r = 10 kDa), Alexa Fluor 555 phalloidin (A34055) and Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (A11001) were purchased from ThermoFisher Scientific (Waltham, MA, USA). Anti-GJA1 antibody (Ab63851) was purchased from Abcam (Cambridge, UK). Apocynin (sc-203321), anti-GAPDH (sc-25778) and horseradish peroxidase-conjugated anti-rabbit antibody (sc-2004) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All drugs were used according to the manufacturer's instructions.

- *Cell cultures*

Primary myometrial cells were obtained from two different sources: a clinical cohort of pregnant women and a commercial source consisting of non-pregnant donors. Access to human samples is difficult and limited, so we also performed experiments on commercial uterine smooth muscle cells (UtSMC). Throughout the manuscript, cells obtained from the clinical cohort will be referred to as "myometrial cells" whereas purchased cells will be called "UtSMC". Concerning the clinical cohort, myometrial cell lines were established from human myometrial biopsies as previously described [18]. Briefly, myometrial biopsies were obtained from pregnant women undergoing elective caesarean section for cephalopelvic disproportion between 38 and 40 weeks and before labor onset. This study was approved by the Comité de Protection des Personnes (CPP) Grand Est (No. CPP:2010/02) and the

Agence Nationale de Sécurité du Médicament et des Produits de Santé (ANSM; 2009-A01176-51), and written informed consent was obtained from all donors. Biopsies were cut into small strips and cultured in 100 mm culture dishes in Dulbecco's modified Eagle's medium (DMEM, Dominique Dutscher, Brumath, France) supplemented with 20% fetal bovine serum (FBS, Dominique Dutscher, Brumath, France) and 1% PSA (100 IU/mL Penicillin G, 100 µg/mL Streptomycin and 0.250 µg/mL Amphotericin B, Dominique Dutscher, Brumath, France). After 2 weeks, cells reached confluence. The explants were gently removed; cells were harvested with trypsin-EDTA, centrifuged at 240g for 10 min at room temperature (RT), resuspended in DMEM supplemented with 10% FBS at the required density and placed into new culture dishes. Concerning the commercial source, primary UtSMC were obtained from Lonza (CC-2562, Basel, Switzerland) by enzymatic dispersion and cultured according to the manufacturer's instructions. All the experiments were performed on myometrial cells from the third to the sixth passage. Human monocytes were purified from peripheral blood mononuclear cells (PBMC) obtained from blood donor buffy coats (provided by Etablissement Français du Sang, Besançon, France) by two-step density gradient centrifugation, as previously described [19]. Monocytes were then differentiated into macrophages by culture with GM-CSF (100ng/ml) for 7 days in Roswell Park Memorial Institute medium (RPMI, Dominique Dutscher, Brumath, France) supplemented with 10% FBS and 1% PSA. Frozen THP1 immortalized cell lines purchased from American Type Culture Collection (ATCC TIB-202, Manassas, VA, USA) were cultured according to the manufacturer's instructions in RPMI medium supplemented with 10% FBS and 1% PSA. THP1 cells were differentiated using 200nM PMA for 72 hours. PMA treatment was followed by 5 days resting in culture without PMA to enhance differentiation features in a pattern similar to monocyte-derived macrophages, as previously demonstrated [20]. Throughout this article, cells obtained from blood donor buffy coats will be referred to as "PBMC-derived macrophages" whereas cells obtained from THP1 immortalized line will be called "THP1-derived macrophages". If not specified, cells used in the co-culture were myometrial cells from patient biopsies and PBMC-derived macrophages.

- *Stimulation protocol*

Myocytes were seeded in 6-well plates for Western blotting (10^6 cells per well), 24-well plates for immunofluorescence and scrape loading/dye transfer assay (2.5×10^4 cells on sterile glass coverslip and 2.5×10^5 cells per well, respectively) and 35mm glass-bottomed dishes for calcium imaging (2.5×10^5 cells per dish). Cells were allowed to adhere for 24h and starved for 48h in serum-free DMEM. Finally, the medium was removed and differentiated macrophages (macrophage:myocyte) were added at a ratio of 1:4 in Opti-MEM (ThermoFisher Scientific). For collagen lattice experiments, myocytes and macrophages were seeded together. Cells were stimulated either with LPS at 100ng/ml [21] or H_2O_2 at 1 μ M in Opti-MEM [13]. The stimulations were done in presence or absence of GSH (100 μ M), apocynin (100 μ M) or catalase (1000 IU/ml) added 1 hour before LPS in Opti-MEM. For each experiment, time-matched vehicle controls (with the same solvent of the stock solution of the drug used for the stimulations) were performed. For Transwell experiments, myometrial cells were cultured in the lower compartment and macrophages were seeded to the upper compartment. Then, LPS (100ng/ml) was added either to the upper or the lower compartment while GSH (100 μ M) was added in the upper compartment.

- Collagen lattices

Myometrial cells or UtSMC, with or without macrophages, were included in collagen gels as described [22]. Briefly, confluent cells harvested with trypsin-EDTA were centrifuged at 1000g for 5 min and resuspended in 10% FBS-DMEM at the required cell density. A rat tail type-I collagen solution (3mg/ml in 0.1N HCl, ThermoFisher Scientific) was adjusted to pH 7.2 with 0.1N NaOH. The final concentration of collagen was 1.5mg/ml. Myometrial cells or UtSMC (1.5×10^5) and PBMC- or THP1-derived macrophages (3.75×10^4) were then added to the neutralized collagen solution. Collagen gel-cell suspensions were incubated in culture dishes (diameter, 35mm) for 1h at 37°C to allow gelling, and then 2ml of fresh 10% FBS-DMEM was added over the cell-collagen lattice. Two days later, the culture medium was replaced with Opti-MEM. The lattices were then gently detached from the sides and lifted off the bottom of the culture dish. The cells in the lattice were then stimulated with LPS (+/- apocynin or catalase) or H_2O_2 . Images of the floating gels were captured before adding the test agents and then every day for up to 5 days and digitized using ChemidocTM XRS+ (BioRad,

Hercules, CA, USA). The areas of the lattices were measured with Image J software, and the percentage of collagen contraction was calculated by comparing the 96h surface area to the basal surface area. The measurements were taken from at least three separate experiments.

- *Western blotting*

After removing the medium, cells were lysed in 100µl of cold RIPA buffer [40mM Tris-HCl (pH 7.4), 150mM NaCl, 2mM EDTA, 1mM sodium orthovanadate, 50mM sodium fluoride, 1% Triton X100, 1% protease and phosphatase inhibitors (Sigma Aldrich)]. Total protein content was determined by Nanodrop (ThermoFisher Scientific); samples were dissolved in Laemmli 5X buffer [312.5mM Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, 0.01% bromophenol blue, 25% β-mercaptoethanol] and boiled for 3 min at 95°C. Thirty micrograms of total protein were loaded per well and subjected to SDS-PAGE before being transferred to a polyvinylidene fluoride membrane. To block nonspecific antibody binding sites, membranes were incubated for 1 h in 5% non-fat dried milk powder in tris-buffered saline with Tween 20 (TBST; 10mM Tris, 150mM NaCl (pH 7.4) and 0.1% Tween 20) solution at RT. Blots were probed overnight at 4°C with rabbit anti-GJA1 primary antibody (1/500) in 1% non-fat dried milk powder in TBST. After three washes with TBST, blots were incubated for 1h at RT with horseradish peroxidase-conjugated anti-rabbit antibody (1/10000) and washed three times with TBST. Immunoreactive proteins were detected by enhanced chemiluminescence (Santa Cruz Biotechnology). Briefly, the membranes were incubated for 1 min in ECL and then exposed to Kodak film for 5 minutes. The film was then digitized using GS-800 densitometer and the intensities of the bands were analyzed densitometrically with QuantityOne software (BioRad). The blots were stripped and reprobed with GAPDH antibody (1/1000) used as a loading control. Results were expressed as means +/- standard error of the mean (SEM) in arbitrary density units (ADU).

- *Immunofluorescence analysis*

Myometrial cells were plated on coverslips in 24-well dishes and then transferred to serum-free DMEM for 48h. If necessary, macrophages were added to the myometrial cells and all cells were then stimulated with LPS (+/- apocynin or catalase) or H₂O₂ for 24h. Cells were fixed in 4% formaldehyde for 5 min followed by permeabilization with 0.1% saponin in phosphate-buffered saline with Tween

20 (PBST; 137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄ (pH 7.4) and 0.1% Tween 20) for 10 min. Actin was visualized using Alexa Fluor 555 phalloidin (1/80) while vinculin was stained using mouse monoclonal anti-vinculin antibody (1/100), followed by incubation with a Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (1/1000) as a secondary antibody. Nuclei were stained with 4-6 diamidino-2-phenylindole (DAPI, Sigma-Aldrich) at 100ng/ml for 3 min. Coverslips were rinsed twice in PBS and mounted onto superfrost slides in a Dako anti-fading mounting medium (Dako, S3023, Carpinteria, CA, USA). Slides were viewed on an Eclipse E400 fluorescence microscope (Nikon, Tokyo, Japan). Three representative pictures were taken in random chosen fields. Pictures were analyzed and merged. Phalloidin staining was quantified by measuring mean fluorescence intensity using Image J software (National Institutes of Health, Bethesda, MD, USA). Results were expressed as means +/- SEM in ADU.

- *Calcium imaging*

Changes in Fura-2 fluorescence were used to measure intracellular Ca²⁺ concentration. Briefly, myometrial cells were plated in 35mm glass-bottomed dishes and transferred to serum-free DMEM for 48h. Macrophages were then added if needed, and all cells were stimulated with LPS or H₂O₂ for 24h. After treatment, cells were loaded with 1μM Fura-2-acetoxymethyl ester (ThermoFisher Scientific) for 15 min at RT in the dark. After loading, the cells were rinsed twice with PBS and incubated an additional 30 min at RT to allow for Fura-2 ester hydrolysis. Measurements were made on an inverted microscope (Axiovert 40 CFL, Carl Zeiss Microscopy GmbH, Jena, Germany) attached to a dual-excitation spectrofluorometer with excitation wavelengths alternating between 340 and 380 nm. The 340/380 nm ratio (F340/380 ratio) was calculated from background-subtracted images and determined for 10 cells in random chosen fields in response to a brief pulse of oxytocin (100nM). F340/F380 ratio was used for quantitative evaluation of changes in the cytosolic calcium concentration. Average ratio values were reported as a function of time and the area under the curve (AUC) was calculated for each condition.

- *Scrape loading/dye transfer assay*

GJ activity of control and treated cells was analyzed using the scrape loading/dye transfer method (SL/DT) as previously reported [23]. Briefly, myometrial cells were seeded in 24-well plates. Macrophages were then added if needed. Following treatment, the cells were cut with a surgical scalpel in the presence of LY (0.05%) and RD (2.5 mg/mL), and then incubated for 3 min at RT. Cells were rinsed three times with PBS. LY and RD fluorescence was visualized with an inverted fluorescence microscope (Station Cell Observer, Carl Zeiss Microscopy GmbH, Jena, Germany). RD was used as a control to label the initially loaded cells and remained confined to the wounded cells bordering the scratch, whereas LY can be transferred to adjacent, unperturbed cells via open GJ channels. The distances of LY diffusion after scrape loading were compared among the different treated groups. Three experiments were carried out for each treatment and three representative pictures were taken for each experiment. Pictures were then analyzed and merged using Image J software (National Institutes of Health, Bethesda, MD, USA).

- *Statistical analysis*

The differences between three or more groups were determined by one-way ANOVA or two-way ANOVA, followed by Bonferroni's multiple comparison test. Comparisons between two groups were made with individual t-tests. Statistical analysis was carried out using GraphPad InStat software (San Diego, CA, USA). Statistically significant results between groups were defined as $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).

Results

- *Macrophages are required for LPS-induced human myometrial contraction*

We first assessed the requirement of macrophages on human myometrial cell contraction after LPS stimulation using collagen lattices. The complete contraction of collagen lattices was only obtained when myometrial cells were co-cultured with PBMC-derived macrophages and stimulated with LPS (Figures 1A to 1C). Indeed, the percentage of collagen contraction was 37% in vehicle-treated condition and 44% after 96h of LPS stimulation, for myometrial cells alone (Figure 1B, P =non significant [NS]). In comparison, the contraction of collagen gel was 37% in vehicle-treated condition

and 62% in LPS-stimulated condition when myometrial cells were co-cultured with macrophages (Figure 1B, $P < 0.01$). Figure 1C shows that the myocyte contraction induced by LPS in the co-culture condition started 24 hours post-stimulation. When compared with the vehicle-treated condition, the area of the collagen lattices was significantly decreased 72 and 96 hours after LPS stimulation (Figure 1C, $P < 0.05$ and $P < 0.01$, respectively). These results indicate that macrophages promote LPS-induced contraction of myometrial cells.

- *Setting up the co-culture model for the study of myometrial labor-associated mechanisms*

Given how difficult it is to obtain human samples, we investigated whether UtSMC could be used in our co-culture model to study labor-associated mechanisms. However, since UtSMC come from non-pregnant donors, we investigated whether the pregnancy-related origin of myometrial cells was important for our co-culture model. We compared the response to LPS of myometrial cells and UtSMC when co-cultured with human macrophages. Contraction experiments showed a similar response to LPS stimulation regardless of myocyte origin (Supp Figures 1A, 1B). These data demonstrated that primary UtSMC can be used in our co-culture model to study myometrial contraction.

Furthermore, since obtaining macrophages from PBMC (from blood buffy coats) is a long and expensive process for our co-culture model, we wondered whether macrophages derived from the THP1 monocytic cell line could be a simpler alternative. We therefore tested the response of myometrial cells to LPS when they were co-cultured with human macrophages obtained either from PBMC or from the THP1 cell line. As shown in Supplemental figure 2, we observed differences between PBMC- and THP1-derived macrophage responses in control conditions rather than in LPS-stimulated conditions. Indeed, the response of vehicle-treated myometrial cells co-cultured with THP1-derived macrophages was excessive, blunting the effect of LPS on myometrial contraction (Supp Figures 2A, 2B). These data suggest that the use of THP1-derived macrophages may not be appropriate for the study of myometrial labor-associated mechanisms, especially collagen contraction assays. As expected, the behavior of primary PBMC-derived macrophages towards an inflammatory stimulus was more physiological than that of an already established monocytic cell line.

- *Different contribution of macrophages to uterine differentiation stages*

Since myometrial cell differentiation precedes contraction, we sought to investigate the involvement of macrophages in the early differentiation steps: i) cytoskeleton reorganization, ii) functional GJ formation and iii) excitability, which are required to induce full contractions.

In myometrial cells with or without the presence of macrophages, we evaluated the ability of LPS to induce actin stress fibers as an indicator of cytoskeleton reorganization. When myometrial cells were cultured alone, actin stress fibers were generally distributed in parallel arrays along the length of the cells, both in vehicle-treated and LPS-stimulated conditions (Figure 2A). In contrast, when co-cultured with macrophages, LPS stimulation led to enhanced myometrial cell spreading, representative of a laboring myocyte profile, with actin stress fibers that extended the entire length of the cell aligned in a nonparallel manner. Moreover, vinculin colocalized with actin at the ends of those filaments, which reflects the formation of focal adhesion sites (Figure 2A, white arrows) [24,25]. Finally, LPS stimulation in presence of macrophages induced the formation of actin stress fibers (Figure 2B, $P < 0.001$).

Next, we investigated the formation of functional GJ between myometrial cells. This step is essential for efficient uterine contractions, and it involves an increase in the expression of GJ channels. We evaluated gap junction intercellular communication (GJIC) function using the GJ permeable fluorescent dye LY by the SL/DT assay. As shown in figure 2C, LPS favored LY transfer to adjacent cells via open GJ channels, only in co-culture conditions, demonstrating that macrophages were needed for LPS-induced myometrial intercellular communication. In contrast, the 10-kDa RD remained confined to the cells at the wound edge in which the dye had been directly introduced during the scrape-loading process. To further determine whether the change in GJIC level by LPS in the presence of macrophages was related to an up-regulation of GJ protein expression, we evaluated the expression of GJA1 (the major GJ protein found in human myometrium) in myometrial cells cultured alone and in our co-culture model (Figure 2D). Densitometric analysis of the bands revealed that

myometrial expression of GJA1 was increased in response to LPS stimulation in the presence of macrophages (Figure 2E, $P < 0.05$).

Finally, we evaluated whether macrophages were required for myometrial excitability assessed by calcium imaging. As presented in figure 2F, LPS induced a similar increase in calcium influx in myometrial cells both when cultured alone and with macrophages. Compared to controls, the AUC of LPS conditions were similarly increased for the myometrial cells cultured alone and in co-culture (Figure 2G, $P < 0.001$). This result suggests that the presence or absence of macrophages does not interfere with myometrial excitability by LPS.

- *Preliminary activation of macrophages by LPS is essential for myometrial cell differentiation*

We further characterized the interaction between myometrial cells and macrophages using a Transwell system that made it possible to stimulate one or the other cell type in the upper (macrophage) or lower (myocyte) compartment. Figure 3A shows that direct LPS stimulation of myometrial cells (i.e. addition in the lower compartment) failed to induce actin reorganization. Actin stress fibers retained their distribution in parallel arrays along the length of myometrial cells. In sharp contrast, when LPS was added to the upper compartment containing macrophages, we observed enhanced cell spreading through actin reorganization and colocalization of vinculin staining at the ends of actin fibers in the myometrial cells seeded in the lower compartment, similar to what we observed when the two cell types were cultured in direct contact (Figure 3A, white arrows). Quantification of the phalloidin staining also demonstrated that LPS stimulation of macrophages induced actin fiber formation in myometrial cells (Figure 3B, $P < 0.05$). When LPS was added to the lower compartment, LY transfer through the myometrial cell layer was abrogated, in comparison with the experiments in which LPS was added to the upper compartment or when the two cell types were in direct contact (Figure 3C). Since inflammation is consistently described as being closely associated with oxidative stress, we hypothesized that ROS could be released by macrophages to induce the onset of myometrial contraction and differentiation. We thus pre-incubated macrophages with GSH, a potent antioxidant agent, before LPS stimulation in the upper compartment of the Transwell system. GSH prevented

myometrial actin/vinculin colocalization and actin fiber formation induced by LPS (Figures 3A, 3B). The same results were observed for LY diffusion (Figure 3C). These results demonstrated that previous activation of macrophages by LPS is essential for the differentiation of myometrial cells and that macrophage/myocyte interactions involve the macrophage release of ROS.

- *Uterine contraction and differentiation require macrophage-released ROS*

To better characterize the involvement of ROS released by macrophages in myometrial cell contraction and differentiation, we pre-incubated co-cultures with apocynin or catalase before LPS stimulation. We observed that NADPH oxidase inhibition with apocynin fully abrogated LPS/macrophage-induced myometrial contraction (Figures 4A, 4B, $P < 0.01$) and differentiation (Figures 4C to 4E, $P < 0.01$). Similarly, these myometrial events induced by LPS were also antagonized by catalase, the enzyme that catalyzes hydrogen peroxide decomposition (Figures 4A to 4E, $P < 0.01$). These data strengthen the hypothesis that ROS (superoxide anion and hydrogen peroxide) play a role in labor, especially hydrogen peroxide signaling because of its greater half-life and its ability to cross biologic membranes.

- *H₂O₂ induces contraction and differentiation of myometrial cells*

Finally, to know more about the macrophage-released ROS involved in myometrial contraction and differentiation, we stimulated myometrial cells directly with H₂O₂, the stable and reactive end-product of superoxide anion dismutation. Contrary to what was observed following direct stimulation with LPS (Figure 1), H₂O₂ stimulation induced collagen lattice contraction in myometrial cells cultured alone (Figure 5A, left panel). When compared with the basal surface area, the percentage of contraction observed after 96 hours of stimulation with H₂O₂ was 65% and only 37% for the vehicle-treated condition (Figure 5B, $P < 0.01$). The result with H₂O₂ in myometrial cells alone was similar to that observed in co-culture conditions after LPS stimulation (Figure 5A right panel, Figure 5C, 65% for H₂O₂ in myocytes alone *versus* 62% for LPS in co-culture, $P = \text{NS}$). Furthermore, H₂O₂ stimulation activated myocyte cell spreading, actin/vinculin colocalization (Figure 5D, white arrows), actin fiber

formation (Figure 5E) and GJIC of myometrial cells (Figure 5F), similar to what we observed in our co-culture model after the addition of LPS.

Discussion

Uterine macrophage infiltration and the subsequent secretion of pro-inflammatory cytokines have been widely described in laboring women and rodents and appear to be crucial for labor onset [7–9,11,26]. Nevertheless, few studies have investigated the precise role of the infiltrating macrophages or their crosstalk with myocytes. In this study, we demonstrated that macrophages are required for the LPS-induced myometrial differentiation into a contractile phenotype and for the subsequent contractions. Furthermore, we highlighted that these processes are mediated by macrophage-released ROS. This observation allows us to suggest a mechanism by which macrophages interact with myometrial cells, and it provides a biological explanation for the presence of infiltrated macrophages. Figure 6 provides a schematic summary of the macrophage-myometrial cell co-culture effects demonstrated in this study.

- *Role of macrophages in myometrial cell contraction and differentiation*

While human studies solely reported macrophage infiltration of myometrium in laboring women [7–9,11,26], macrophage depletion experiments in mice implicated macrophages in LPS-induced preterm labor but did not explore their function [11]. We demonstrated, both in vitro and on human primary cells, the critical interaction between macrophages and myometrial cells in LPS-induced labor mechanisms. Indeed, we showed that myometrial contractions in response to LPS were significantly weaker in the absence of macrophages.

Since labor is the terminal differentiation phase of uterine myocytes [14], we focused on the differentiation steps and explored whether the role of macrophages was limited to the contractile feature of labor onset. At term, signals that initiate labor ultimately promote a switch in the phenotype of the quiescent uterus to a smooth muscle which becomes spontaneously excitable and exhibits a high degree of cell-cell coupling [14]. This study demonstrates that macrophages were required for cytoskeleton reorganization and functional GJ formation. LPS stimulation only led to a massive actin reorganization of the myometrial cells when they were co-cultured with macrophages. The dynamic

remodeling and induction of actin fiber formation in myometrial cells contribute to the initiation and maintenance of smooth muscle contraction. Myometrial cells are very rich in actin fibers, which generally appear as parallel bundles along the longest axis of the cells [24]. We also demonstrated that, in the presence of macrophages, LPS stimulation led to the distribution of actin stress fibers in a nonparallel manner that extended the entire length of the cell, colocalizing with vinculin staining at the ends of the filaments. We thus believe that actin filaments, obliquely organized along the entire length of the cell, and actin/vinculin focal adhesion complexes create strong cytoskeletal bridges with the surrounding cells. Moreover, we observed that LPS stimulation in the presence of macrophages led to an increase in LY transfer that was related to an increase in GJA1 expression. GJ are also necessary for parturition, with increased expression of GJA1 and increased abundance and size of GJ in uterine smooth muscle immediately before parturition [27,28]. These changes make it possible for the myometrium to contract synchronously during labor by promoting cell-to-cell transfer of low molecular weight ions and molecules and intercellular electrical coupling. Interestingly, we showed that macrophages were not required for myometrial excitability since LPS stimulation led to a calcium influx in myometrial cells that was independent of the presence of macrophages. The fact that myometrial cells express toll-like receptor 4 (TLR4) [29] may implicate the LPS effect in calcium influx. Moreover, a recent study reported that LPS-induced contractions of uterine myometrium involve RhoA kinase (ROCK)-dependent calcium sensitization after TLR4 stimulation [30]. Altogether, our study demonstrated that macrophages, although not involved in myometrial excitability, contribute to the reorganization of the myometrial cytoskeleton and the formation of functional GJ, two crucial differentiation steps for labor, especially for the initiation and maintenance of coordinated and directed contractions.

Finally, our results offer an explanation for the apparently contradictory results obtained from in vitro and in vivo studies of myometrial response. For example, intra-amniotic infusion of IL-1 β to pregnant non-human primates induced parturition within 48 hours [31], but in vitro studies failed to demonstrate the role of IL-1 β in myometrial cell contraction [32] and activation [33]. The lack of immune cells such as macrophages within the in vitro set up may explain these discordant results,

underscoring the interest of using co-culture models like ours for the study of labor-associated mechanisms.

- *Involvement of macrophage-released ROS in myometrial contraction and differentiation*

The second key finding of this work is that macrophage-induced oxidative stress is involved in myometrial contraction and differentiation. First, we demonstrated that previous activation of the macrophages was essential for myometrial cell differentiation, suggesting sequential interaction between macrophages and myocytes. When macrophages were cultured in Transwell inserts, their stimulation by LPS led to the reorganization of the cytoskeleton and GJIC in the myometrial cells seeded in the compartment below, similar to what occurred when the cells were co-cultured in direct contact. These results suggest that macrophages release intermediary messenger(s), potentially oxidative stress molecules and pro-inflammatory cytokines. Our observation that antioxidant agents (glutathione, catalase and apocynin) abrogated myometrial differentiation and contractions induced by the macrophage supernatants suggest that oxidative stress plays a major role in this process. Indeed, we demonstrated that superoxide anion signaling contributed to LPS-induced labor mechanisms. Nevertheless, our previous work found that the release of IL-1 β by macrophages was mediated by oxidative stress, suggesting that the role of cytokines should not be disregarded [13].

Additionally, we demonstrated that H₂O₂ mimicked the effects of the LPS-stimulated macrophage supernatants in myometrial cell differentiation, thereby providing a mechanistic explanation for the requirement of macrophages for labor. Although inflammation is a well-studied feature of PTL pathophysiology, one of the least explored areas is oxidative stress, an inseparable component of inflammation. While excessive ROS are toxic, moderate levels of ROS can serve as signaling intermediates, regulating physiological cellular processes including growth, immune defense and migratory response [34]. Oxidative damage due to the excessive generation of ROS has been linked to the development of various diseases, including cardiovascular disease, cancer, chronic inflammation, and neurologic disorders [35]. Although little is known about the implication of ROS in labor, some data suggest that oxidative stress plays a role in PTL pathophysiology [36–39]. An increase in ROS

production has been demonstrated in circulating monocytes from patients with preterm labor compared with gestation-matched non-laboring women [5], and MacIntyre et al. [40] reported that LPS-induced PTL involved the activation of AP-1, which is a redox-sensitive transcription factor. It would seem that chronic oxidative stress, along with DNA damage and cell senescence, leads to preterm premature rupture of the membranes (pPROM) while acute oxidative stress is associated with spontaneous preterm birth (PTB). A recent study revealed that oxidative stress-induced DNA damage and senescence observed in fetal membranes have characteristic features of pPROM rather than PTB with intact membranes [39]. The differences in oxidative stress-induced pathways and responses could further differentiate the mechanisms that lead to PTB and pPROM [37,39].

- *Development of a co-culture model to study labor-associated mechanisms*

Our team developed a novel co-culture model of myometrial cells and macrophages for the purposes of this study. Previous studies have investigated the interactions between myometrial cells and monocytes [41,42]. Since contact between monocytes, myocytes and the microenvironment lead to differentiation into macrophages, our co-culture model could be a valuable tool for the in vitro study of labor-associated mechanisms. The use of primary cells for both myocytes and macrophages is also important seeing as it contributes to obtaining cellular behavior that is as close as possible to the physiological response. Concerning myocytes, one of the advantages highlighted by our experiments is the possibility of using UtSMC, which expands the use of our co-culture model to researchers with limited access to human samples. Indeed, the origin of the myometrial cells from biopsies of pregnant or non-pregnant women had no influence on the study of labor-associated contractions. On the contrary, we demonstrated that the use of THP1-derived macrophages led to an excessive response of vehicle-treated conditions. Such cells did not appear to be appropriate under our co-culture conditions (cell number and ratio with myocytes), but further variations of some co-culture parameters might lead to their effective use.

This is a preliminary study that was used to establish an effective co-culture model. Therefore, one of the limitations of our work is that our model does not fully mimic the mechanisms of labor, because

these mechanisms are artificially induced by LPS. However, the LPS stimulation conditions reproduce the features observed in pathological human tissues obtained during chorioamnionitis [21]. Furthermore, several inflammatory signals can induce significant reorganizations of the uteroplacental sphere. We would like to evaluate the robustness of our model by testing other inflammatory signals (infectious or sterile), especially IL-1 β or TNF α , two cytokines that play a major role in labor onset [31]. The co-culture model utilized in this work may be useful in different studies in the field of labor, for instance to explore pathophysiological pathways or for the screening of new tocolytics. Current tocolytic therapies have limited efficacy against myometrial contractions. Therefore, studies investigating the underlying mechanisms leading to preterm labor are critical to identify new potential therapeutic targets. Furthermore, this co-culture model can be seen as a promising alternative to animal models for drug development in humans. Our data validate a novel alternative cell co-culture approach to rapidly prioritize efficacious preventive or therapeutic agents in humans and, thereby, reduce and refine traditional animal studies identifying interventional strategies for future clinical trials on PTL prevention or management.

In summary, this study demonstrates that LPS-activated macrophages transactivate myometrial cells through ROS production, resulting in myometrial differentiation (except for excitability induction) and subsequent contractions. Our findings strongly suggest the need for innovative PTL management strategies based on the use of antioxidants agents and the inhibition of initial laboring stages, before the acquisition of the contractile phenotype. Macrophages could represent a new therapeutic target in the pharmacological management of preterm labor, and future research on prematurity could be extended towards macrophage biology.

Author's contribution

All authors contributed to the writing and editing of this manuscript, and approved the final version. MW, TH, MP, MB, TL, FN performed the experiments. PS was in charge of patient recruitment and myometrium biopsy collection. MW, CG and FL contributed to the design of the study, data analysis and interpretation of the data.

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Conflict of interest

The authors state no conflict of interest.

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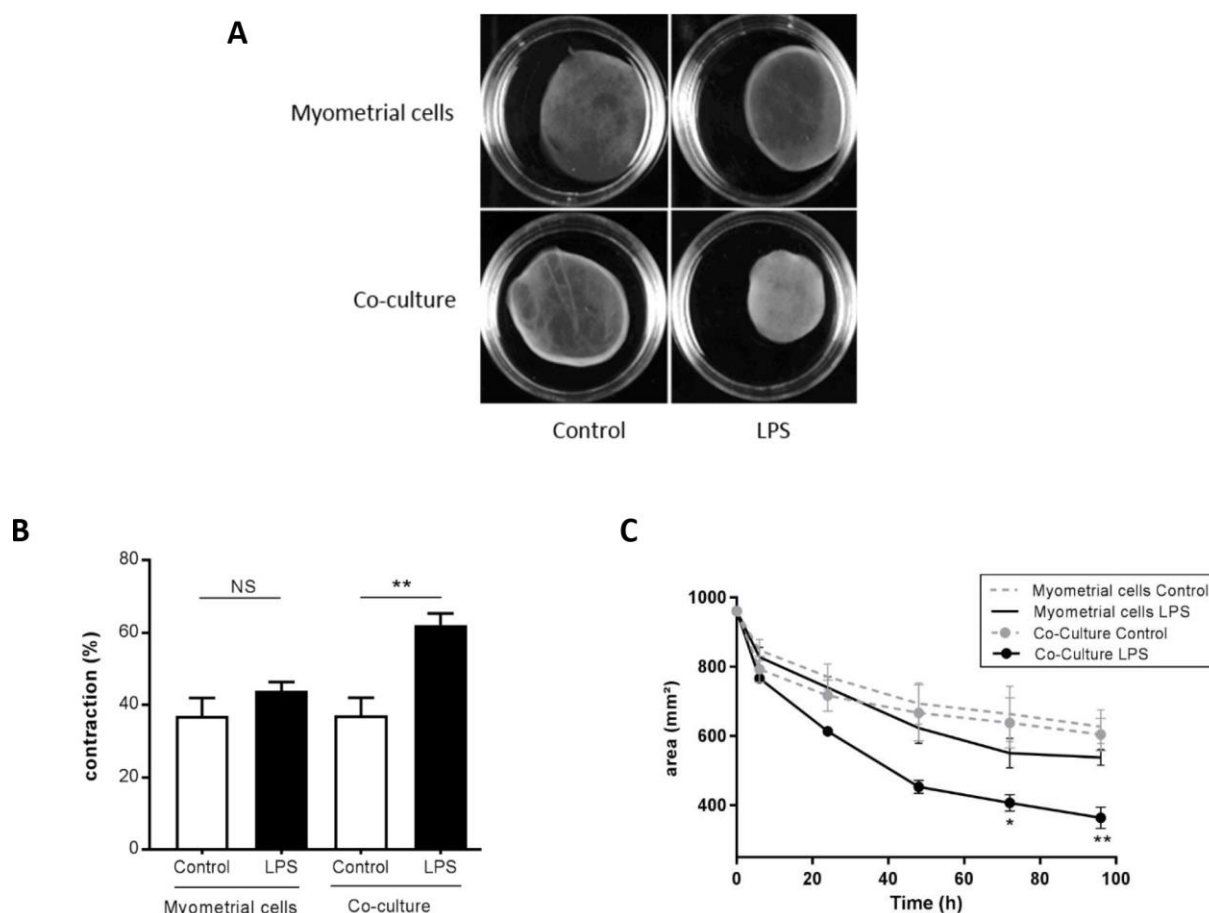


Figure 1: Macrophages are required for LPS-induced myometrial contraction. **A)** Photographs of collagen lattices of either myometrial cells alone (upper row) or co-cultured with PBMC-derived macrophages (lower row), in presence or absence of LPS (100ng/ml) for 96h. Images shown are representative of six experiments. **B)** Mean percentage contraction of collagen lattices \pm SEM after 96h of stimulation with LPS, compared to the basal surface area, $n=6$, NS=non significant, $**P<0.01$. **C)** Time-dependent variation of collagen lattices area \pm SEM up to 96h of stimulation with LPS (100ng/ml). $*P<0.05$ and $**P<0.01$ versus Co-culture Control.

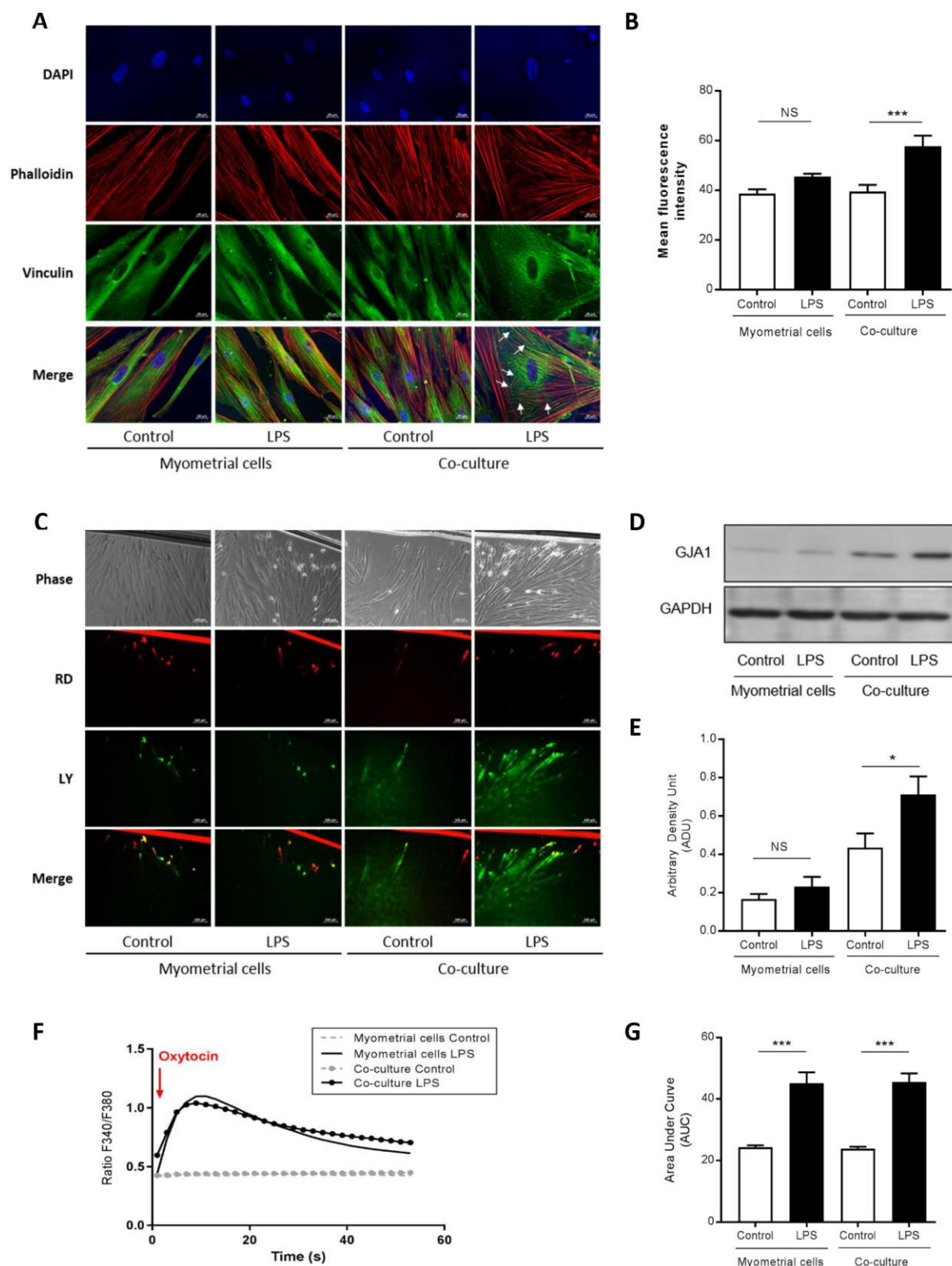


Figure 2: Role of macrophages in LPS-induced uterine differentiation stages. Myometrial cells were cultured alone or co-cultured with macrophages in presence or absence of LPS. **A)** Fluorescence images of the actin cytoskeleton (red), vinculin (green) and DAPI for nuclear localization (blue).

White arrows represent complexes formed by vinculin at the ends of the actin filaments. Pictures were taken with an epifluorescence microscope at x1000 magnification in random chosen fields and are representative of three pictures for each condition from six experiments. **B)** Bar chart showing mean fluorescent intensity of phalloidin stained actin cytoskeleton (mean \pm SEM, n=6, NS=non significant, ***P<0.001). **C)** Phase contrast images (upper row) and fluorescence images of RD (red) and LY (green) transfer to adjacent myometrial cells after scrape loading. Pictures were taken with an inverted microscope at x200 magnification in random chosen fields and are representative of three experiments. **D-E)** Representative blots and semi-quantification of GJA1 and GAPDH protein levels. Densitometric analysis is represented as fold induction (mean \pm SEM) and expressed in ADU, n=9, NS=non significant, *P<0.05. **F)** Time-dependent variation of the cytosolic calcium concentration evaluated by the F340/F380 ratio in myometrial cells in response to a brief pulse of oxytocin (100nM). For each condition, measurements were made for 10 cells in random fields and average ratio values are representative of five experiments. **G)** Quantification of calcium imaging. The AUC is represented as mean \pm SEM, n=5, ***P<0.001.

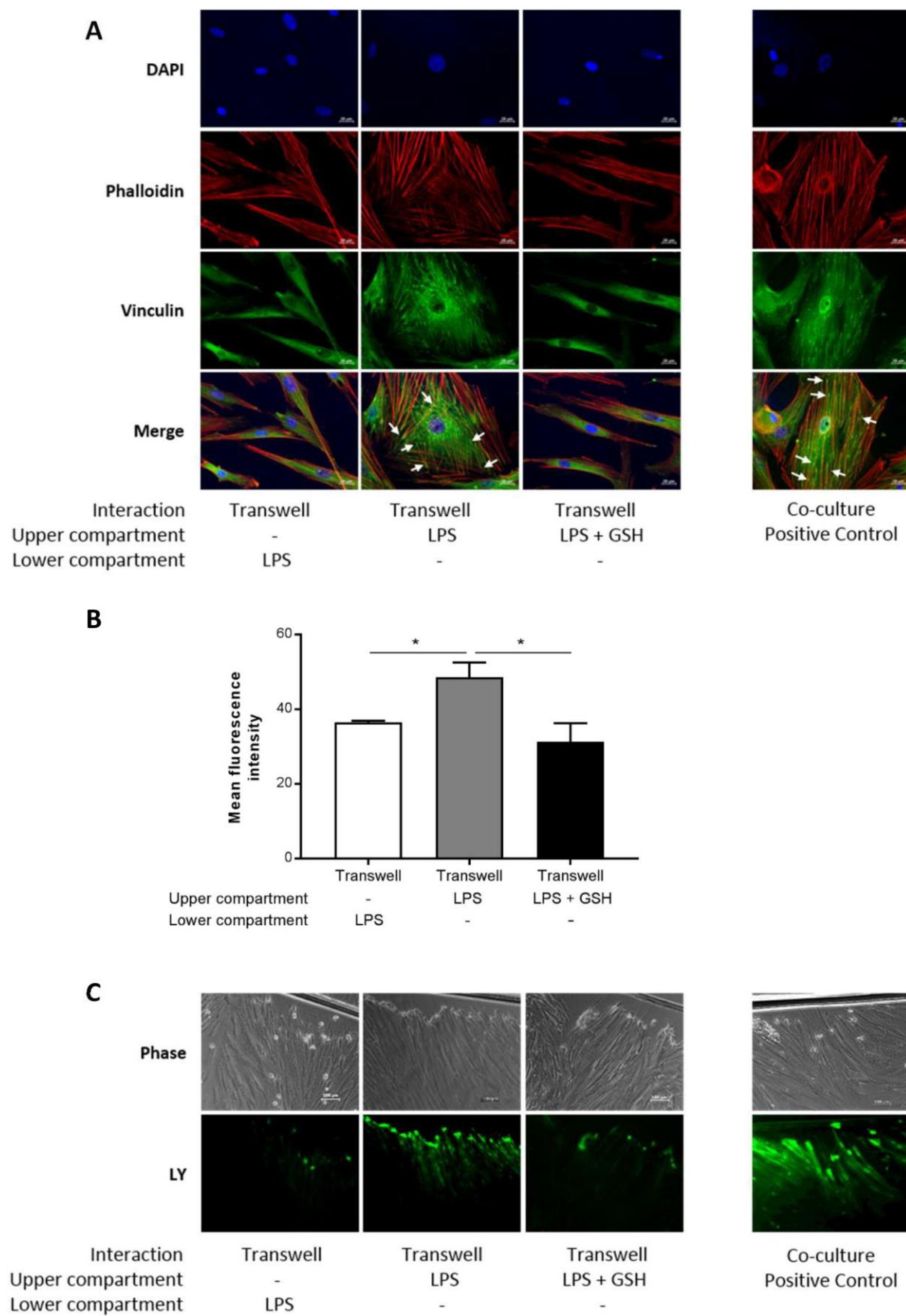


Figure 3: Preliminary activation of macrophages by LPS is essential for the differentiation of the myometrial cells. Transwell experiments with macrophages (upper compartment) or myocytes (lower compartment) following stimulation by LPS alone (100ng/ml) or LPS + GSH (100μM). On the right column, pictures represent a positive control in which macrophages and myocytes were co-cultured in direct contact. **A)** Fluorescence images of the actin cytoskeleton (red), vinculin (green) and DAPI for nuclear localization (blue). White arrows represent complexes formed by vinculin at the ends of the actin filaments. Pictures were taken with an epifluorescence microscope at x1000 magnification in random chosen fields and are representative of three pictures for each condition from three experiments. **B)** Bar chart showing mean fluorescent intensity of phalloidin stained actin cytoskeleton (mean ± SEM, n=3, *P<0.05). **C)** Phase contrast images (upper row) and fluorescence images of LY (green) transfer to adjacent myometrial cells after scrape loading. Pictures were taken with an inverted microscope at x200 magnification in random chosen fields and are representative of three experiments.

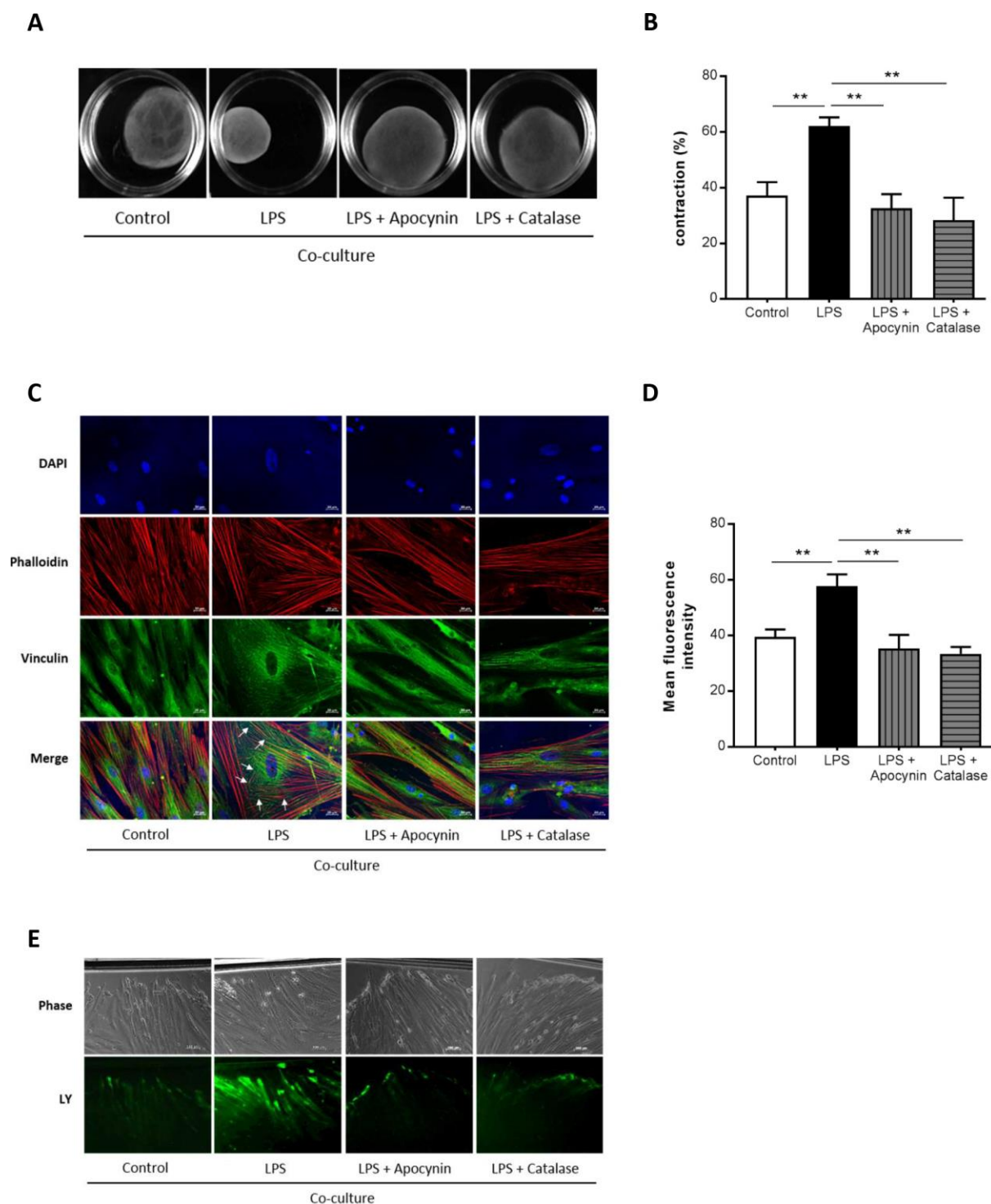


Figure 4: Myometrial contraction and differentiation require macrophage-induced oxidative stress. Co-cultures of myometrial cells and macrophages were pre-incubated with apocynin (100 μ M) or catalase (1000 IU/ml) before LPS stimulation (100ng/ml). **A)** Photographs of collagen lattices at 96h post-stimulation are representative of three experiments. **B)** Mean percentage contraction of collagen lattices \pm SEM at 96h post-stimulation, compared to the basal surface area, $n=3$, $**P<0.01$.

C) Fluorescence images of the actin cytoskeleton (red), vinculin (green) and DAPI for nuclear localization (blue). White arrows represent complexes formed by vinculin at the ends of the actin filaments. Pictures were taken with an epifluorescence microscope at x1000 magnification in random chosen fields and are representative of three pictures for each condition from four experiments. **D)** Bar chart showing mean fluorescent intensity of phalloidin stained actin cytoskeleton (mean \pm SEM, n=4, **P<0.01). **E)** Phase contrast images (upper row) and fluorescence images of LY (green) transfer to adjacent myometrial cells after scrape loading. Pictures were taken with an inverted microscope at x200 magnification in random chosen fields and are representative of three experiments.

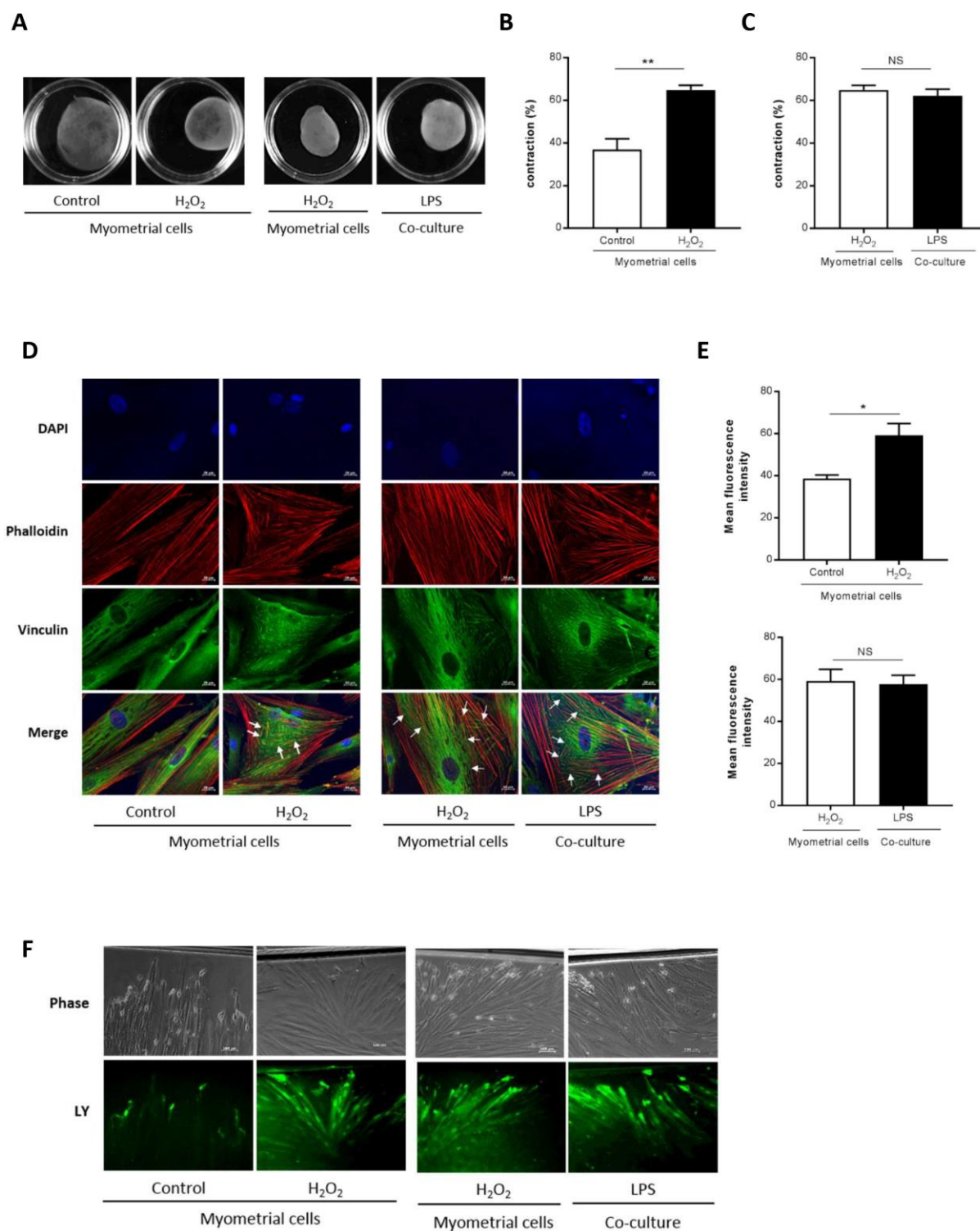


Figure 5: H_2O_2 direct effect inducing contraction and differentiation of myometrial cells. Myometrial cells were stimulated or not with H_2O_2 ($1\mu M$) whereas co-cultures of myometrial cells and macrophages were stimulated with LPS ($100ng/ml$). **A**) Photographs of collagen lattices at 96h post-stimulation are representative of six experiments. **B-C**) Mean percentage contraction of collagen lattices \pm SEM at 96h post-stimulation, compared to the basal surface area, $n=6$, NS=non significant,

****P<0.01. D)** Fluorescence images of the actin cytoskeleton (red), vinculin (green) and DAPI for nuclear localization (blue). White arrows represent complexes formed by vinculin at the ends of the actin filaments. Pictures were taken with an epifluorescence microscope at x1000 magnification in random chosen fields and are representative of three pictures for each condition from six experiments. **E)** Bar chart showing mean fluorescent intensity of phalloidin stained actin cytoskeleton (mean \pm SEM, n=6, NS=non significant, *P<0.05). **F)** Phase contrast images (upper row) and fluorescence images of LY (green) transfer to adjacent myometrial cells after scrape loading. Pictures were taken with an inverted microscope at x200 magnification in random chosen fields and are representative of three experiments.

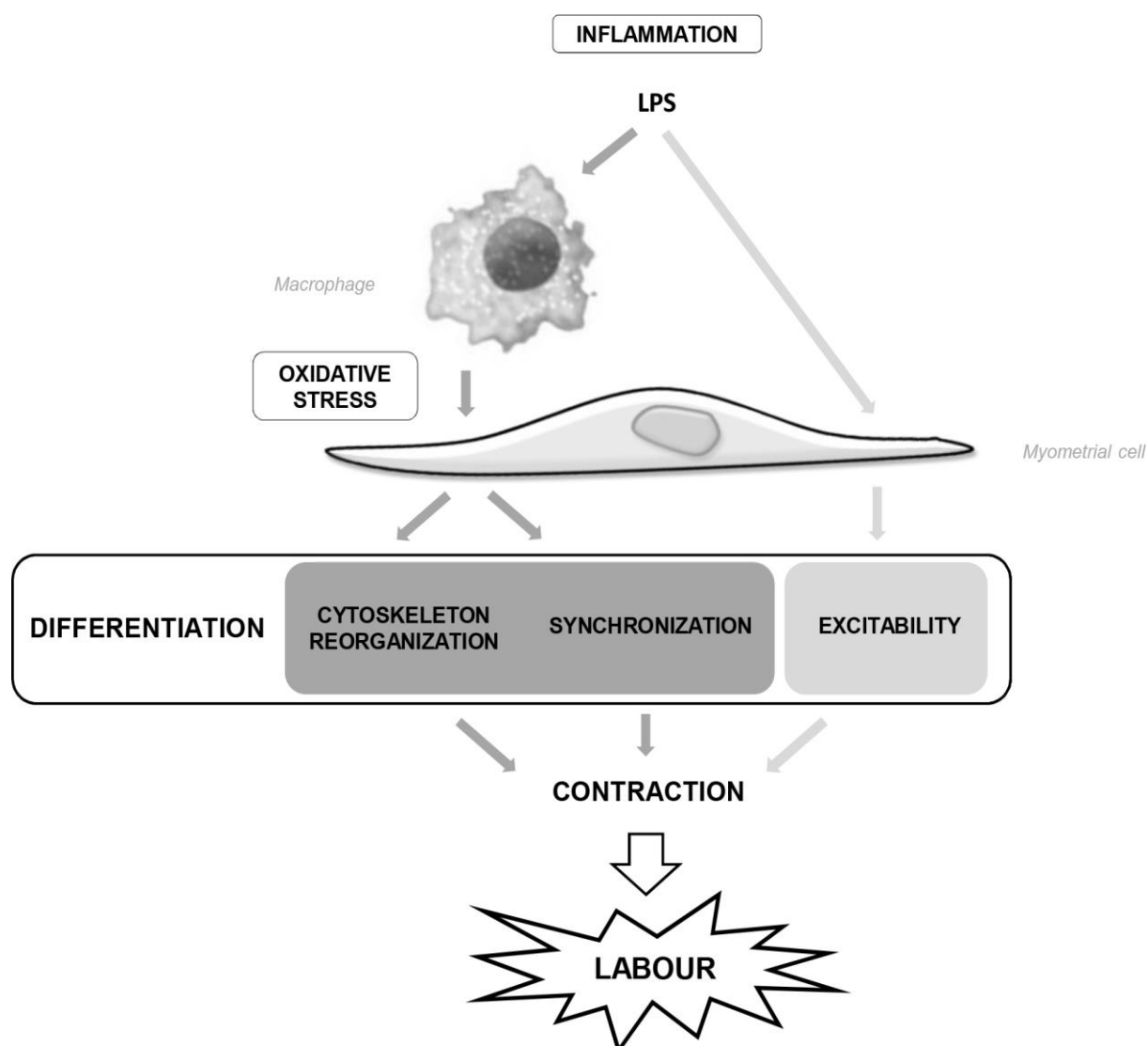


Figure 6: Scheme outlining the role of macrophages on myometrial cells in our LPS-induced co-culture model. LPS treatment of myometrial cells co-cultured with PBMC-derived macrophages leads to cytoskeleton reorganization and functional GJ formation, indirectly through LPS-induced macrophage ROS release. Full myometrial differentiation is achieved with enhanced myocyte excitability, induced directly by LPS.