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Abstract: Microparticles (MPs) are membrane fragments that may play a role in the pathogenesis of chronic respiratory diseases. We aimed to investigate whether human monocytes/macrophage-derived MPs could induce a pro-inflammatory phenotype in human bronchial smooth muscle cells (BSMC) and the effect of montelukast in this setting. Experimental methods included isolation of human monocytes/macrophages and generation of monocyte-derived MPs, RT-PCR analysis of gene expression, immunoenzymatic determination of pro-inflammatory factor release, bioluminescent assay of intracellular cAMP levels and electromobility shift assay analysis of NF-κB nuclear translocation. Stimulation of human BSMC with monocyte-derived MPs induced a pro-inflammatory switch in human BSMC by inducing gene expression (COX-2 and IL-8), protein release in the supernatant (PGE2 and IL-8), and heterologous β2-adrenoceptor desensitisation. The latter effect was most likely related to autocrine PGE2 since pre-treatment with COX inhibitors restored the ability of salbutamol to induce cAMP synthesis in desensitised cells. Challenge with MPs induced nuclear translocation of NF-κB and selective NF-κB inhibition decreased MP-induced cytokine release in the supernatant. Montelukast treatment prevented IL-8 release and heterologous β2-adrenoceptor desensitisation in human BSMC exposed to monocyte-derived MPs by blocking NF-κB nuclear translocation. These findings provide evidence on the role of human monocyte-derived MPs in the airway smooth muscle phenotype switch as a novel potential mechanism in the progression of chronic respiratory diseases and on the protective effects by montelukast in this setting.

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Editorial Office
PHARMACOLOGICAL RESEARCH

Dear Editor,

I am submitting the manuscript by Fogli, S., *et al*. entitled *"Montelukast prevents microparticle-induced inflammatory and functional alterations in human bronchial smooth muscle cells"* for possible publication as an original article in the Pharmacological Research. All coauthors have given their approval for the submission of the present work and no substantial part of this manuscript has been published or is considered for publication elsewhere.

I would be pleased to provide any additional information and integration if needed. I wish to thank you for your time and kind attention.

Sincerely yours,

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Montelukast prevents microparticle-induced inflammatory and functional alterations in human bronchial smooth muscle cells

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Running title: Montelukast and microparticles

Abstract

Microparticles (MPs) are membrane fragments that may play a role in the pathogenesis of chronic respiratory diseases. We aimed to investigate whether human monocytes/macrophage-derived MPs could induce a pro-inflammatory phenotype in human bronchial smooth muscle cells (BSMC) and the effect of montelukast in this setting. Experimental methods included isolation of human monocytes/macrophages and generation of monocyte-derived MPs, RT-PCR analysis of gene expression, immunoenzymatic determination of pro-inflammatory factor release, bioluminescent assay of intracellular cAMP levels and electromobility shift assay analysis of NF-κB nuclear translocation. Stimulation of human BSMC with monocyte-derived MPs induced a pro-inflammatory switch in human BSMC by inducing gene expression (COX-2 and IL-8), protein release in the supernatant (PGE2 and IL-8), and heterologous β2-adrenoceptor desensitisation. The latter effect was most likely related to autocrine PGE2 since pre-treatment with COX inhibitors restored the ability of salbutamol to induce cAMP synthesis in desensitised cells. Challenge with MPs induced nuclear translocation of NF-κB and selective NF-κB inhibition decreased MP-induced cytokine release in the supernatant. Montelukast treatment prevented IL-8 release and heterologous β2-adrenoceptor desensitisation in human BSMC exposed to monocyte-derived MPs by blocking NF-κB nuclear translocation. These findings provide evidence on the role of human monocyte-derived MPs in the airway smooth muscle phenotype switch as a novel potential mechanism in the progression of chronic respiratory diseases and on the protective effects by montelukast in this setting.

Key words: microparticles, Montelukast, β2-agonists, airway smooth muscle cell, bronchial inflammation, cell signaling.

1. Introduction

The mononuclear phagocyte system is a key element in the complex inflammatory network that characterizes asthma and plays an important role in initiating the neutrophilic inflammatory response in patients with chronic obstructive pulmonary disease [1]. Microparticles (MPs) are membrane fragments shed by virtually all eukaryotic cells upon activation o during apoptosis that play a significant role in intercellular communication and also in several pathophysiological processes including coagulation and inflammation [2]. We demonstrated that human monocyte/macrophage-derived MPs up-regulate inflammatory mediator synthesis by human airway epithelial cells [3,4] and elicit an autocrine activation loop by inducing superoxide anion production, cytokine release and NF-κB activation [5]. However, it is unknown whether human airway smooth muscle plays a role in the pro-inflammatory action of monocyte/macrophage-derived MPs in the airways. This represents an interesting point to be investigated because this tissue has important synthetic functions and is a rich source of cytokines, chemokines, inflammatory mediators, and growth factors that contribute to the airway inflammation and remodeling [1].

Montelukast (MK) is a potent cysteinyl leukotriene receptor (CysLTR) antagonist generally considered to have limited application in the therapy of asthma (i.e., exercise- and aspirin-induced asthma and as add-on therapy in patients poorly controlled with inhaled corticosteroids, alone or in combination with β2-adrenoceptor agonists). Convincing evidence has emerged for the possible use of high-dose MK as anti-inflammatory agent in acute asthma [6]. The mechanisms by which MK can elicit these secondary CysLTRs-independent effects have been recently reported [7] suggesting that this agent may have a broader spectrum of anti-inflammatory activities than originally thought.

The present study was aimed at characterizing the pro-inflammatory profile of MPs in BSMC of human origin and the effect of montelukast in this experimental setting. We demonstrate

that human monocytes/macrophages shed MPs that can up-regulate the synthesis of pro-inflammatory mediators by human airway smooth muscle cells and montelukast can reverse this phenotype.

2. Materials and methods

2.1 Generation of monocyte-derived MPs

Human monocytes/macrophages (from which MPs were generated) were isolated from the buffy coats of blood donor by Ficoll gradient centrifugation followed by overnight culture of the mononuclear cell fraction. MPs generation was obtained from adherent cells stimulated with the calcium ionophore, A23187, as previously described [4]. Briefly, human monocytes (about 2 x 10^6 cells/well) were stimulated by the calcium ionophore A23187 at 12 μM in a final volume of 0.5 ml for 20 min at 37°C and the supernatant was recovered and cleared by centrifugation (14000 x g, 5 min, room temperature).

MPs were characterized by multiparametric flow cytometry analysis (BD FACSCalibur™ Software; Becton Dickinson, San Jose, CA) of the monocyte markers CD14 and CD16, using a blend of fluorescent beads (Sigma-Aldrich, USA) of three diameters (0.1, 0.5 and 1 mm), to better identify the gate for MPs. In addition, according to Bardelli and co-workers [5], phosphatidylserine (PS) expression (about 2 nM) and Tissue Factor (TF) activity (9824 ± 1700 A.U.; n= 6) were also evaluated.

Human bronchial smooth muscle cells (BSMC; Lonza, Walkersville, MD, USA) were maintained exactly as recommended by the manufacturer in an optimized medium containing 5% fetal bovine serum, 5.5 mM glucose, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, 5 ng/ml insulin, 2 ng/ml basic fibroblast growth factor and 0.5 ng/ml epidermal growth factor (SmGM-2 Bullet Kit, Lonza).

2.2 RT-PCR analysis

RNA from cells was extracted by using the RNeasy Mini kit and reverse-transcribed by the QuantiTect Reverse Transcription kit. PCR was performed by the Hot StartTaq Master Mix kit. Primers used were: 5'-GCCTGACTCCTTCAAGATCG-3' (F) and 5'-AGGGACAGGTCTTGGTGTTG-3' (R), for COX-1; 5'-TGAAACCCACTCCAAACACA-3' (F) and 5'-AACTGATGCGTGAAGTGCTG-3' (R) for COX-2; 5'-ATGACTTCCAAGCTGGCCGT-3' (F) and 5'-CCTCTTCAAAAACTTCTCCACACC-3' (R) for IL-8; 5'-ACAAGGACGCCATCAACTG-3' (F) and 5'-AAAGACCATAACCACCAAGGG-3' (R) for β2-adrenoceptor; 5'-GTGAAGGTCGGAGTCAACG-3' (F) and 5'-GGTGAAGACGGCCAGTGGACTC-3' (R) for GAPDH and the expected amplification products were 446, 385, 285, and 300 bp long, respectively. Densitometric assay of RT-PCR bands was performed by using the 'Quantity One' softhware (Bio-Rad Laboratories, Hercules, CA, USA).

2.3 ELISA assays

Soluble IL-8 expression was assessed in the conditioned medium of MP-stimulated BSMC with a sandwich ELISA (Hu IL-8 Cytoset, BioSource, Nivelles, Belgium) according to the manufacturers' instructions.

2.4 cAMP assay

Heterologous β2-adrenoceptor desensitisation was tested by exposing human BMSC to monocyte-derived MPs for 24 h. Intracellular cAMP levels were measured by the cAMP-Glo™ Assay (Promega, Madison, WI, USA) in control and desensitised cells after stimulation with 10 μM salbutamol, in the presence or absence of Bay 11-7082 0.1-1 μM or montelukast 30 μM for 24 h. BSMC were used between passages 4 and 8.

2.5 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear protein extracts was obtained by using the Nuclear extract Kit (Active Motif, CA, USA) and then incubated on ice with 32P-labelled PPAR oligonucleotide (Perkin Elmer Life Sciences, Boston, USA) in binding buffer. Consensus oligonucleotide for PPAR was 5'-CAAAACTAGGTCAAAGGTCA-3' (Santa Cruz Biotech, Santa Cruz, USA). DNA/protein complex was size fractionated on a non-denaturing 5% polyacrylamide gel in TBE buffer (100 mM Tris-HCl, 100 mM boric acid, 2 mM EDTA) and detected by autoradiography.

The activation of NF-kB induced by MPs was evaluated by measuring its nuclear migration (by electrophoretic mobility shift assay, EMSA), as previously described [5]. Nuclear extracts (5 □g; obtained by using the Nuclear extract Kit, Active Motif, USA) from BSMC were incubated with 2 □g poly (dI-dC) and [^{32}P] ATP-labelled oligonucleotide probe (100,000-150,000 cpm; Promega) in binding buffer (50% glycerol, 10 mM Tris-HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 1 mM dithiothreitol) for 30 min at room temperature. The NF-kB consensus oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC–3') was from Promega. The nucleotide-protein complex was separated on a 5 % polyacrylamide gel in 0.5 x TBE buffer (100 mM Tris-HCl, 100 mM boric acid, 2 mM EDTA) at 150 V on ice. The gel was dried and radioactive bands were detected by autoradiography [5]. Supershift assays were performed using 4 mg of commercial antibodies (anti-NF-kB p65: sc-8008 X from Santa Cruz Biotech, USA; anti-NF-□B p50: ab7549 from Abcam). Densitometric analysis of NF-kB nuclear translocation was also performed, un-stimulated cells being = 1.

2.6 Data analysis and statistics

All experiments were done in triplicate and data were expressed as mean ± SEM. Results were then plotted by Prism software (Graphpad Software, San Diego, CA, USA). Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple comparisons.

3. Results

3.1 Characterization of monocyte-derived MPs

As reported in Figure 1, unstimulated monocytes are 98% $CD14^+$ (Figure 1A). MPs generated from A23187-stimulated monocytes are 95% $CD14^+$ and about 40% $CD16^+$ (Figure 1B), so indicating the pro-inflammatory potential of the ionophore. As known, expression of the marker CD16 distinguishes two monocyte subsets [8]: CD14+/CD16+ monocytes, which release high amounts of pro-inflammatory cytokines, and CD14+/CD16- monocytes, which represent the "anti-inflammatory" subset, characterized by a poor cytokine release.

3.2 Effect of MPs on the COX-2/prostanoid pathway

Human BSMC were stimulated with monocyte-derived MPs over a period of 24 h to examine the effect of this potential pro-inflammatory stimulus on COX expression and release of PGE2 into the supernatant of cultured cells. A graphical summary of RT-PCR results is depicted in Figure 2A following densitometric analysis of COX-2 expression and normalization to the amount of GAPDH mRNA present in the samples. Specifically, a low expression level of 385 bp COX-2 transcript was identified in unstimulated cells, whereas treatment with isolated MPs significantly increased COX-2 mRNA levels (2.08±0.36-fold, as compared to control; $p<0.05$) without affecting COX-1 expression (Figure 2A). Noteworthy, such an effect was also accompanied by the release of PGE2 from BSMC into the culture medium (3.30±0.81-fold, as compared to control; $p<0.01$; Figure 2B).

3.3 Effect of MPs on β2-adrenoceptor function

Following MP stimulation, no effect was observed on the expression of β2-adrenoceptor gene transcription in BSMC (Figure 2A). Responsiveness to acute salbutamol at 10 μM was assessed in cells exposed to monocyte-derived MPs by measuring intracellular cAMP

synthesis. The ability of salbutamol to induce cAMP synthesis in unstimulated BSMC was reduced from 218±25 to 108±19 nM after treatment with MPs ($p<0.01$; Figure 2B).

3.4 Role of the COX/PGE2 pathway in MP-induced β2-adrenoceptor hyporesponsiveness

We next studied the effects of indomethacin (non-selective COX inhibitor) and NS398 (selective COX-2 inhibitor) in this experimental setting to better characterise the role of COX enzymes on MP-induced β2-adrenoceptor desensitization in BSMC. Treatment with indomethacin or NS398 at 1 μM for 24 h completely restored salbutamol response in MP-desensitized cells, at levels comparable to those obtained in unstimulated cells (Figures 3A and 3B). Noteworthy, heterologous β2-adrenoceptor desensitization was induced by treating unstimulated BSMC with exogenous PGE2 at a concentration similar to that released into the supernatant from MP-stimulated cells (i.e, 100 nM) (Figure 3C).

3.5 Effect of MPs on the IL-8 expression

The IL-8 mRNA levels were analysed by RT-PCR after 24 h of vehicle (control) or MPs and compared. A significant increase in IL-8 mRNA was induced by monocyte-derived MPs (Figure 4A). Unstimulated BSMC constitutively express an average of 350±66 pg/ml IL-8 and overnight incubation with isolated MPs caused a 3-fold increase (1048±175 pg/ml; $p<0.001$) of the IL-8 release into the culture medium (Figure 4B).

3.6 Role of NF-κB in MP-induced pro-inflammatory switch

The selective inhibitor of NF-κB, Bay 11-7082, at 1 μM for 24 h, completely blocked the release of IL-8 (Figure 5A) and PGE2 (Figure 5B) in MP-stimulated BSMC without affecting cytokine synthesis in unstimulated cells. Bay 11-7082, at the concentrations used, did not affect cell viability assessed by WST-1 method (data not shown).

EMSA clearly demonstrated that challenge with MPs markedly increased nuclear translocation of NF-κB in BSMC (Figure 6; lanes 4-6) and the effect was quantified by densitometry (graph on the right). Experiments were performed in the presence of specific antibodies against p65 and p50 to confirm the specificity of the assay (see supershift of the protein-DNA complex and the relative densitometric analysis, respectively on the left and on the right of Figure 6).

3.7 Effect of montelukast on MP-induced NF-κB nuclear translocation

Montelukast 30 μM reduced translocation of the p50/p65 complex into the nucleus induced by MPs in BSMC, at levels comparable to those of unstimulated cells (Figure 6). MK, at the concentrations used, did not affect cell viability assessed by WST-1 method (data not shown).

3.8 Effect of montelukast on MP-induced β2-adrenoceptor desensitization and IL-8 release

Montelukast 30 μM restored salbutamol response in MP-desensitised cells at levels similar to those of controls, whereas drug alone did not significantly affect cAMP synthesis (Figure 7A). Montelukast significantly reduced the IL-8 release ($p<0.01$) from MP-stimulated cells without affecting the baseline IL-8 levels (Figure 7B).

4. Discussion

The cellular and molecular mechanisms that regulate the immunomodulatory functions of airway smooth muscle may offer new and important therapeutic targets in treating asthma and COPD. In line with evidence suggesting that human airway smooth muscle may secrete important factors that modulate submucosal airway inflammation [1,9], the findings of the present study suggest a potential novel mechanism that might contribute to initiate and perpetuate airway inflammation (Figure 8). Indeed, we demonstrate that human monocyte/macrophage-derived MPs activate important inflammatory pathways in BSMC

leading to a phenotype switch. Specifically, MPs increase the expression of COX-2 (but not COX-1) and the synthesis of PGE2 in human BSMC, a phenotype commonly observed following exposure of airway smooth muscle to proinflamatory stimuli [10]. This is most likely an autocrine and/or paracrine compensatory mechanism, because PGE2 is a potent bronchodilator and may also modulate the inflammatory milieu by inhibiting smooth muscle cell secretion of chemokines [11]. However, it should be noted that dysregulation of the PGE2 synthesis/response axis was also observed in human pulmonary fibrotic diseases [12] and continuous stimulation by paracrine/autocrine PGE2 promotes heterologous β2-adrenoceptor desensitisation in airway smooth muscle cells [13,14].

Using inhibitors of cyclooxygenase, we found that β2-adrenoceptor desensitization was mediated through BSMC derived COX-2 induced prostaglandins suggesting that activation of the COX-2/prostanoid pathway by human monocyte/macrophages-derived MPs might promote bronchomotor tone dysregulation and altered response to β2-adrenoceptor agonists (Figure 8), a phenotype observed in patients with chronic respiratory diseases who do not adequately respond to this type of drugs (e.g., those with severe asthma and COPD). Noteworthy, autocrine prostaglandin production as potential mechanism of airway smooth muscle β2-adrenoceptor desensitization, has been recently proposed in rhinovirus infected bronchial epithelial cells [15].

Elevated levels of IL-8 were found in the airway of patients with cystic fibrosis and asthma [16]. Furthermore, IL-8 works as a potent chemo-attractant for neutrophil that is the primary effector cell in COPD and severe asthma [1], and there are evidence establishing novel roles for IL-8 in human airway smooth muscle cell contraction and proliferation [16,17]. In the current work, we demonstrated that human monocyte/macrophage-derived MPs induced IL-8 release into the BSMC supernatant and such an effect was associated to an increase in the IL-8 gene transcription (Figure 8). These findings further underlie the importance of MPs as a key element of the interaction between mononuclear phagocyte

system and human airway smooth muscle, a dynamic triangle that could be critical in the pathophysiological network responsible for airway disease progression.

The NF-κB pathway plays an important role in the TNF-α-induced pro-inflammatory phenotype in human airway smooth muscle cells [18]. We clearly showed that activation of the nuclear NF-κB pathway was the common molecular mechanism leading to the up-regulation of PGE2 and IL-8 in MP-stimulated BSMC (Figure 8). In particular, after exposure of human BSMC to MPs, the released p50/p65 complex translocates into the nucleus and activated the transcription of NF-κB-regulated genes, including those coding for COX-2 and IL-8. Enhanced nuclear translocation of NF-κB by monocyte/macrophage-derived MPs seems to be a hallmark of this membrane vesicle's type action because it was also observed in human lung epithelial cells [4] and in human monocytes [5] during paracrine and autocrine stimulation, respectively.

Our findings demonstrated that MK could reverse the pro-inflammatory phenotype induced in BSMC by human monocyte-derived MPs. In particular, MK prevents nuclear translocation of NF-κB and mimics the effect of the selective inhibitor of NF-κB, Bay 11-7082, thus blocking IL-8 and PGE2 release and restoring salbutamol response in stimulated BSMC (Figure 8). Although the molecular mechanism underlying the inhibitory effect of CysLT1 receptor antagonists on NF-kB activation remains unclear, several lines of evidence indicate that it is not mediated by antagonism of the CysLT1 receptor [19-22]. Therefore, together with the recognised ability to blocks the direct effect of cysLTs on bronchial smooth muscles during the early asthmatic response, the anti-inflammatory properties of MK might be useful, particularly in patients with persistent asthma who do not respond or became resistant to standard treatments. Furthermore, our results suggest that MK may have also an effective role in rheumatic diseases where MPs are an important driver of the inflammatory process [2].

MP research and clinical analysis may be improved by using highly standardised protocols for MP isolation, detection and quantification and future research should be aimed at describing more fully the signal transduction pathways activated by MPs. The elucidation of the protein and lipid composition of monocyte/macrophage-derived MPs was beyond the purposes of the current study where our interest was primarily focused on using a standardised method for the induction of a rapid, complete and reproducible MPs release. With regards for this, the advantage of using the calcium ionophore instead of physiological activators was already discussed [3,5]. In this study and in previously published works [4,5], MPs was obtained by treating human monocytes isolated from the peripheral blood of healthy donors with A23187 at the concentration of 12 μM, which represents the EC50 mean value calculated from concentration-release experiments [3]. Characterization of MPs included the assessment of CD14+ and CD16+ elements percentage, tissue factor activity, phosphatidyl serine (PS) equivalents' concentration, and protein concentration in the samples. Specifically, we used 100 μl of supernatants from A23187-challenged monocytes corresponding to 10μg protein concentration (that ensured maximal effects) and to 2 nM equivalents of PS, in agreement with previously published works [5,23,24].

In conclusion, our findings provide evidence that MPs may play a role in the pathogenesis of airway inflammation by inducing a phenotype switch in human BSMC which consist in nuclear translocation of the p50/p65 complex, activation of NF-κB-regulated genes (including IL-8 and COX-2), induction of cytokine release (IL-8 and PGE2) and autocrine (PGE2-mediated) β2-adrenoceptor desensitization. Furthermore, MK may block this novel mechanism, which could be involved in the progression of chronic respiratory diseases.

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Figure legends

Figure 1. Flow cytometry analysis of human monocytes and MPs from monocytes. Figure shows a forward light scatter versus $CD14^+$ (specific monocyte marker) and $CD16^+$ (pro-inflammatory marker) expression dot plot of unstimulated monocytes (A) and MPs generated from A23187-stimulated monocytes (B). This figure is representative of other four experiments.

Figure 2. COX and β2-adrenoceptor gene expression (A), prostaglandin (PG)-E2 release and β2-adrenoceptor function (B) in human BSMC upon overnight incubation with monocyte-derived microparticles (MPs). Data are reported as mean ± standard error of the mean (SEM). *p<0.05, **p<0.01, ***p<0.001 (n=5).

Figure 3. Effects of indomethacin (non-selective COX inhibitor) (A) and NS398 (selective COX-2 inhibitor) (B) on MP-induced β2-adrenoceptor desensitisation in human BSMC. Heterologous β2-adrenoceptor desensitization induced by exogenous PGE2 (C). Data are reported as mean ± standard error of the mean (SEM). *p<0.05, **p<0.01 (n=5).

Figure 4. (A) RT-PCR images and densitometric analyses of interleukin (IL)-8 gene expression and (B) IL-8 release by MP-stimulated human BSMC. Data are reported as mean ± standard error of the mean (SEM). *p<0.05, ***p<0.001 (n=5).

Figure 5. Effects of the selective inhibitor of NF-κB, Bay 11-7082, at 1 μM for 24 h, on the release of IL-8 (A) and PGE2 (B) by MP-stimulated human BSMC. Data are reported as mean ± standard error of the mean (SEM). ***p<0.001 (n=5).

Figure 6. Image and densitometric analysis of nuclear factor (NF)-κB activation in human BSMC stimulated with MPs in the presence or absence of montelukast 30 μM. Lanes 1-3: control, unstimulated BSMC; lanes 4-6: MP-challenged BSMC; lanes 7-9: montelukast (MK) + MPs. Specific antibodies against p65 and p50 subunits were used to confirm the specificity of the assay (see text for further details). Data are reported as mean ± standard error of the mean (SEM). ***p<0.001 (n=5).

Figure 7. Effect of montelukast (MK) on MP-induced β2-adrenoceptor desensitisation (A) and IL-8 release (B) in human BSMC. Data are reported as mean ± standard error of the mean (SEM). *p<0.05, **p<0.01 (n=5).

Figure 8. Overview of the possible molecular mechanisms involved in the pro-inflammatory switch induced by monocyte-derived microparticles (MPs) in human bronchial smooth muscle cells (BSMC) and the protective effect of montelukast in this specific setting.

Figure(s)

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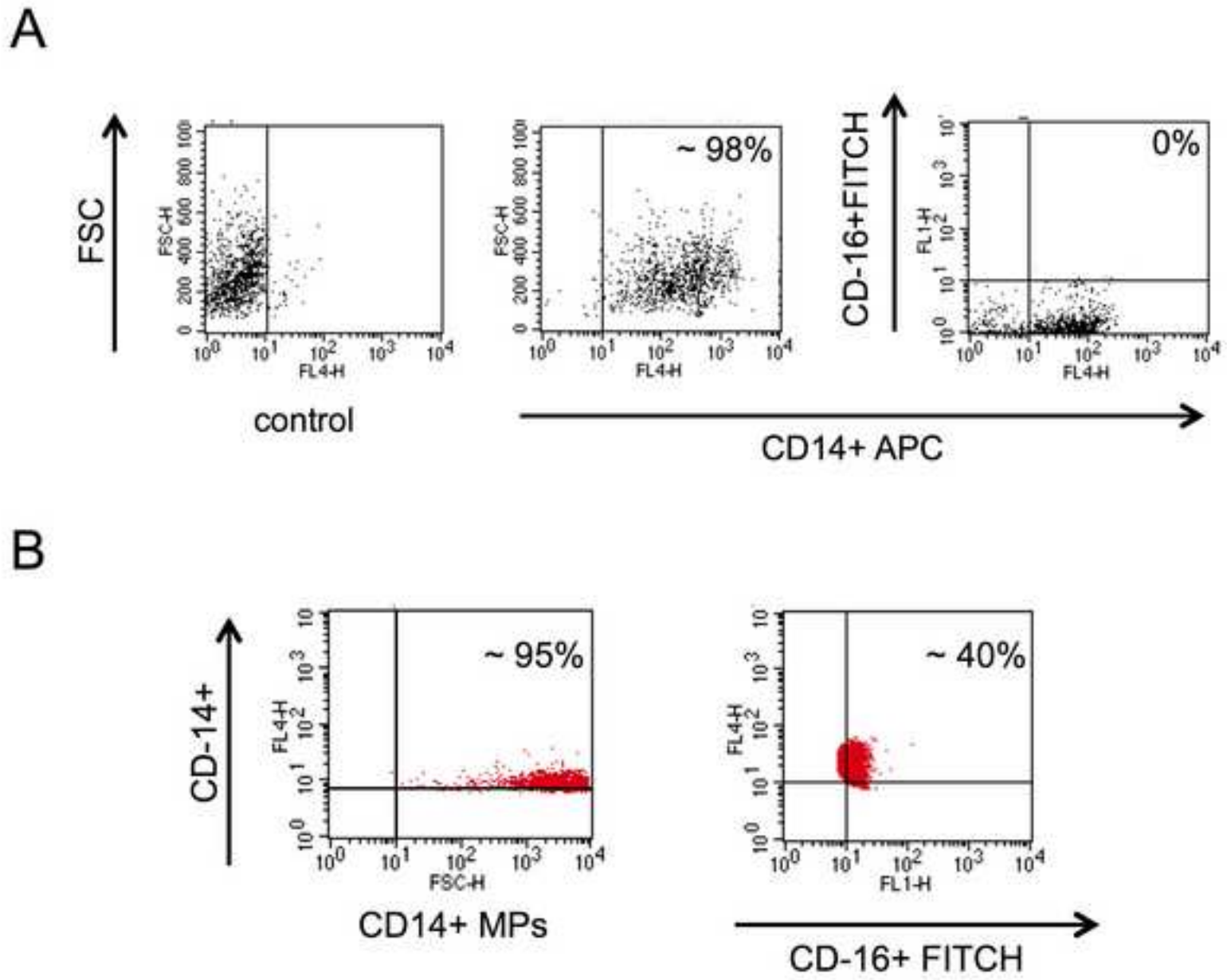

Figure 1

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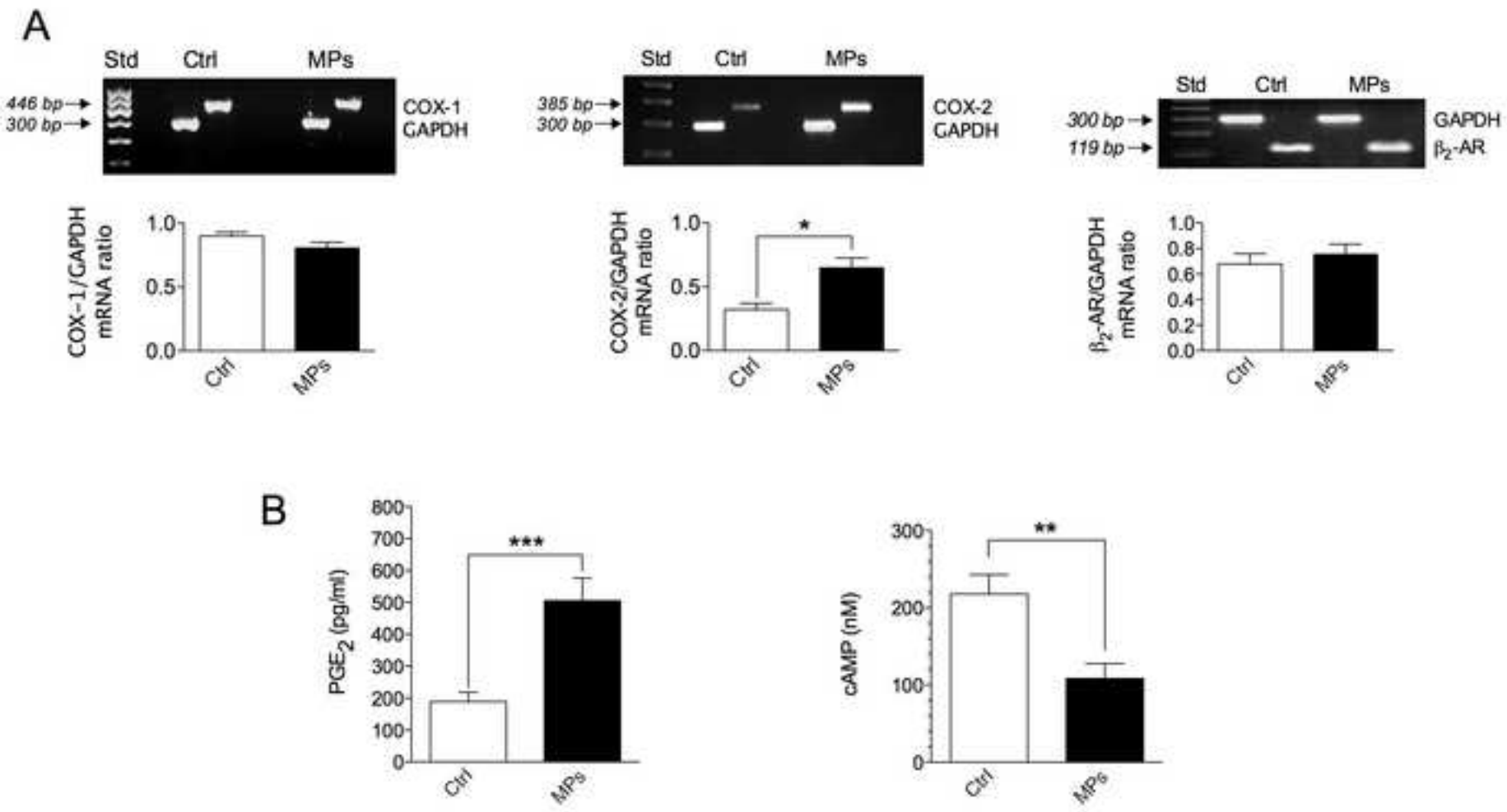

Figure 2

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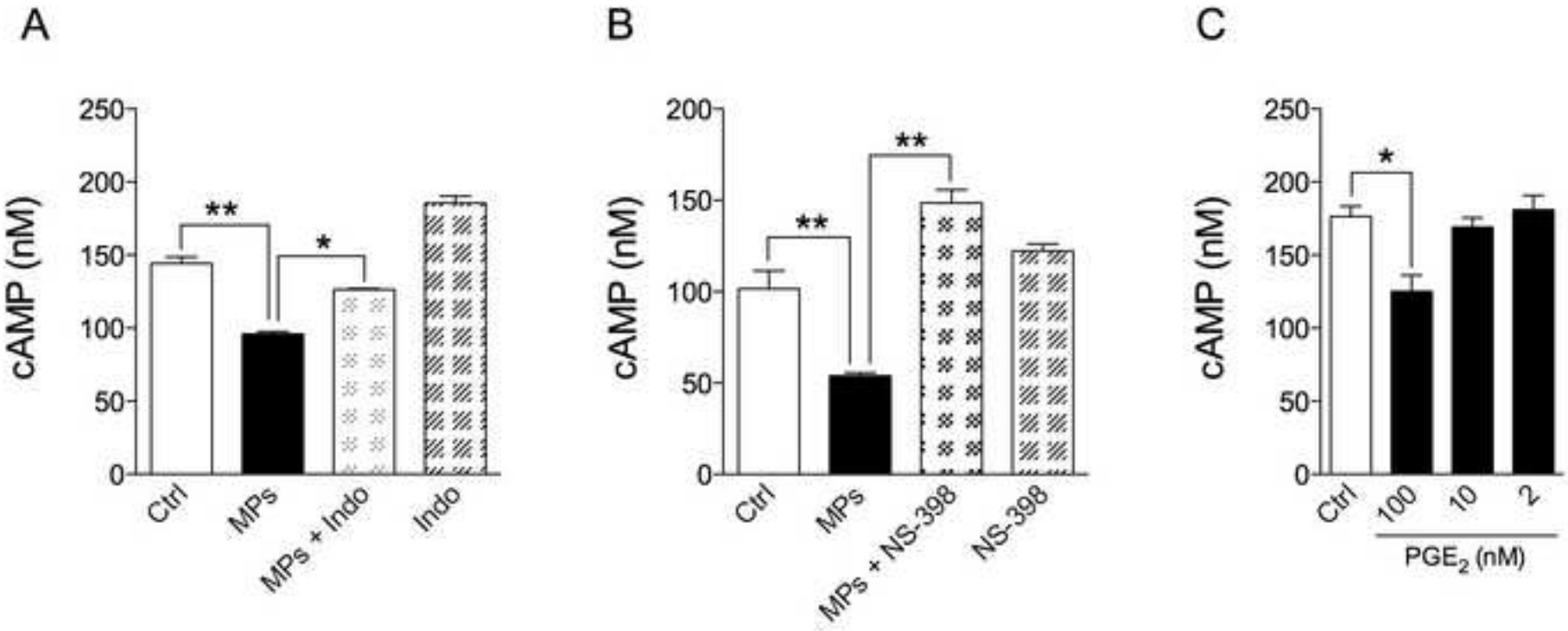

Figure 3

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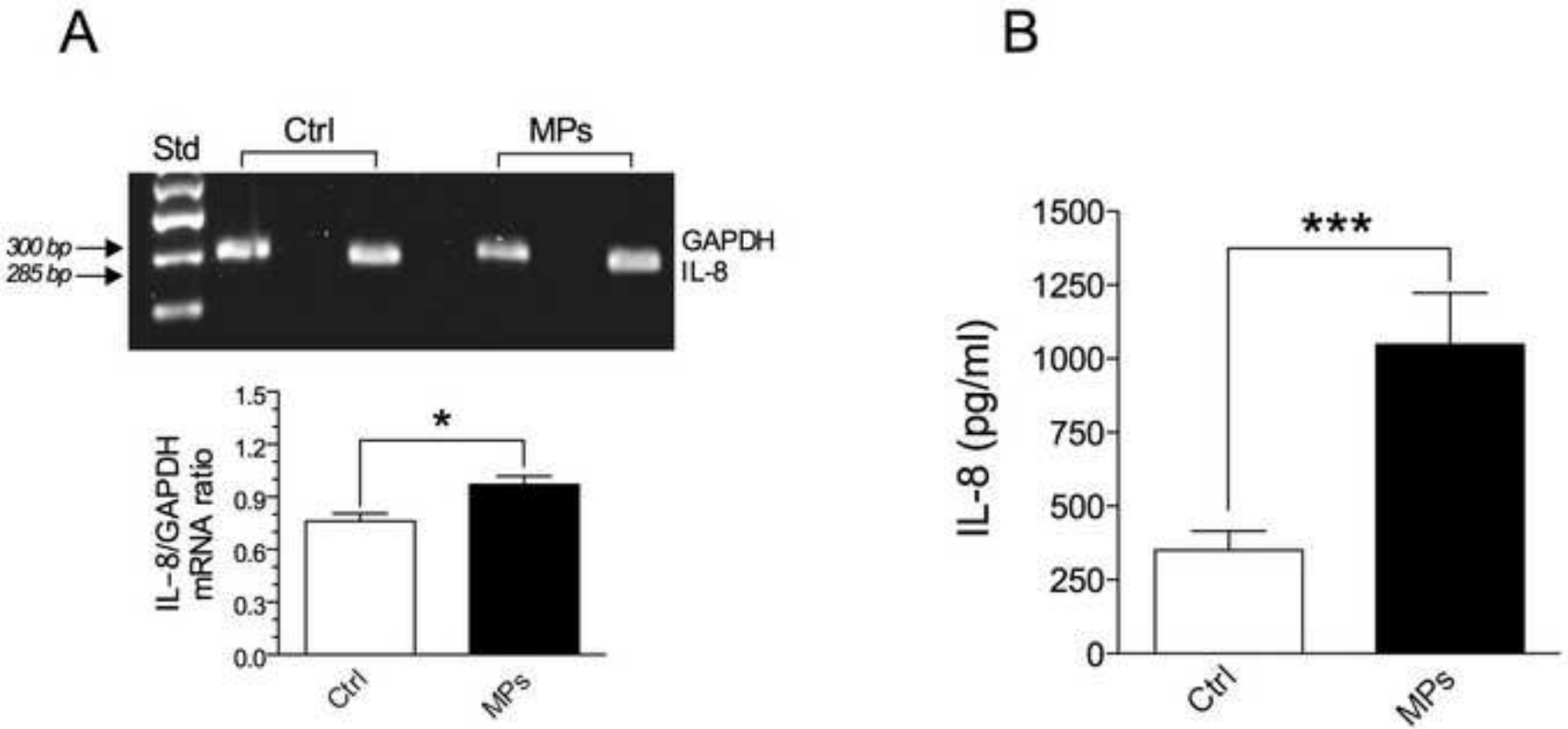

Figure 4

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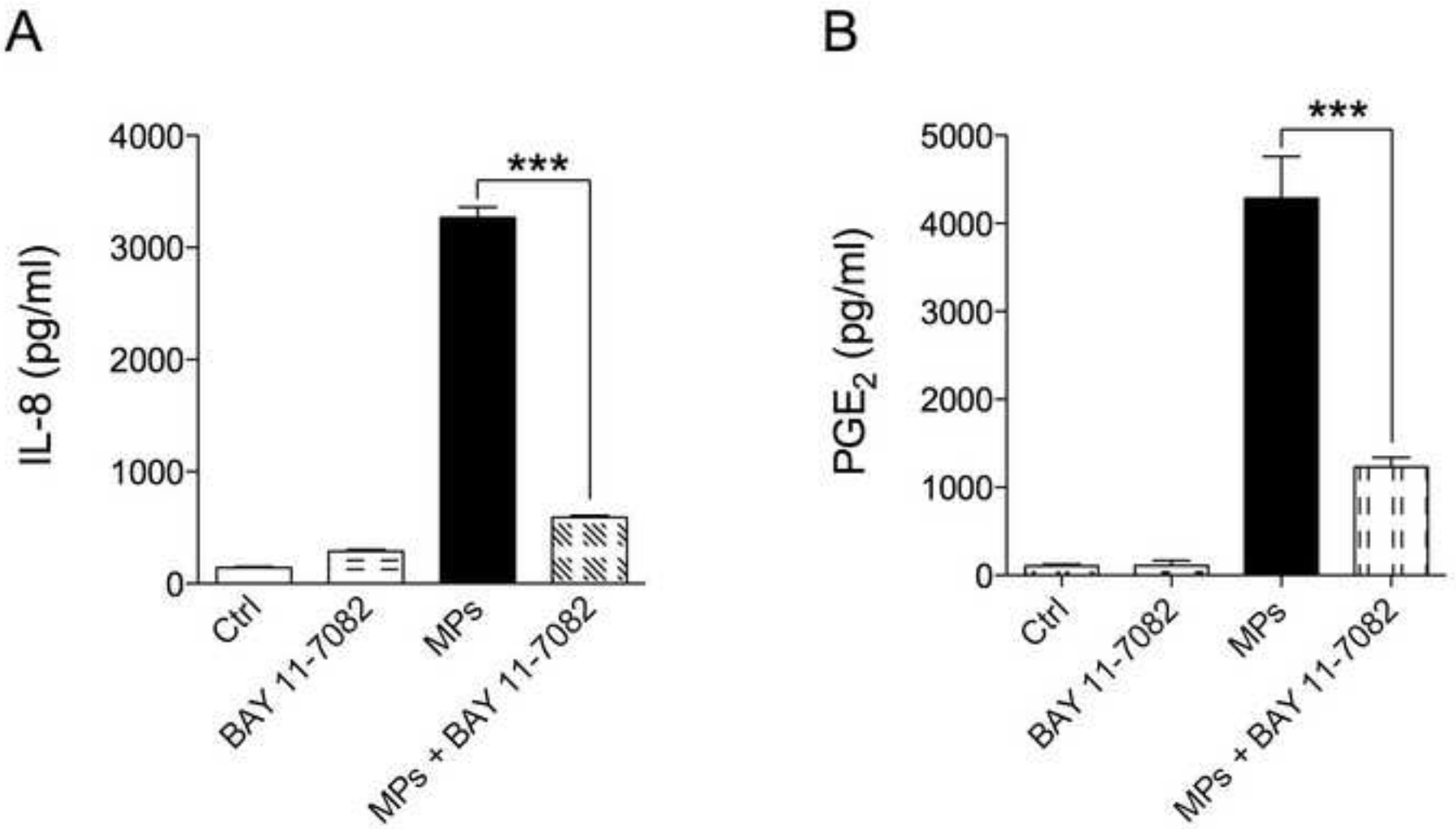

Figure 5

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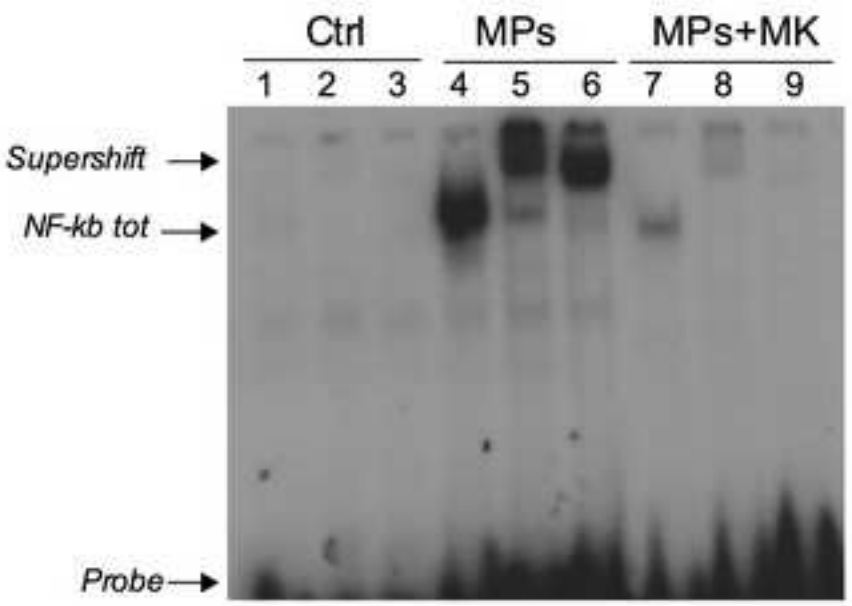

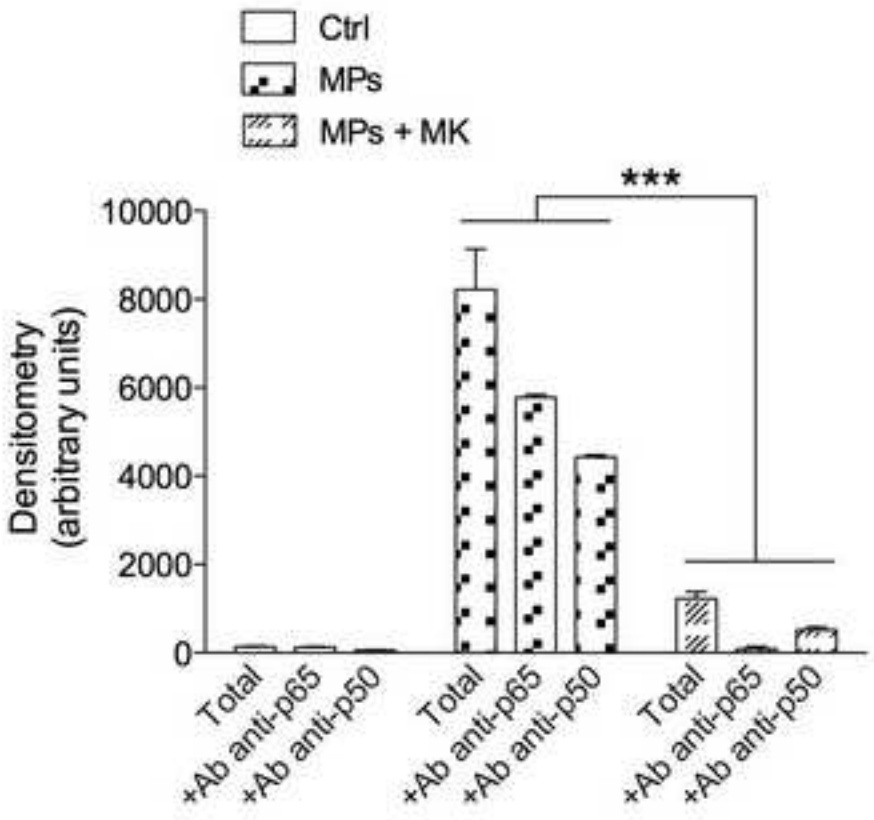

Figure 6

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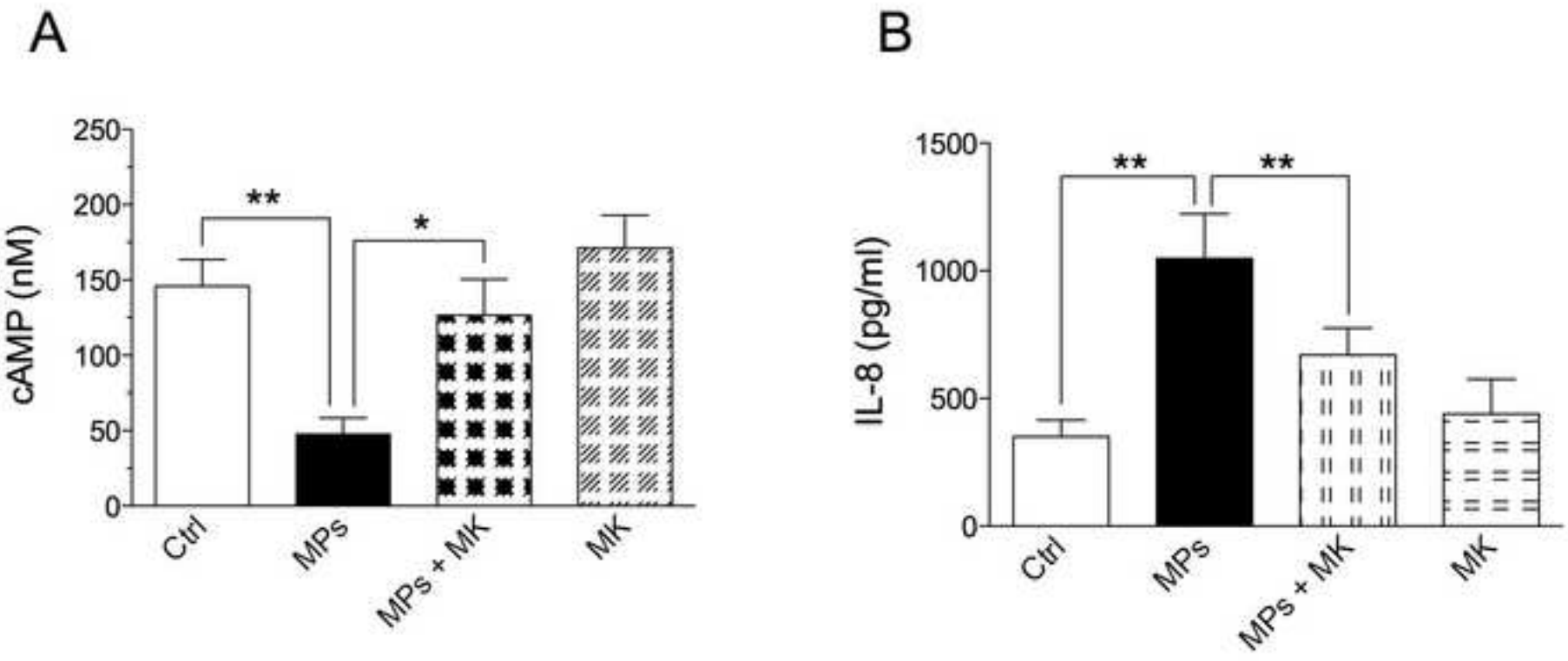

Figure 7

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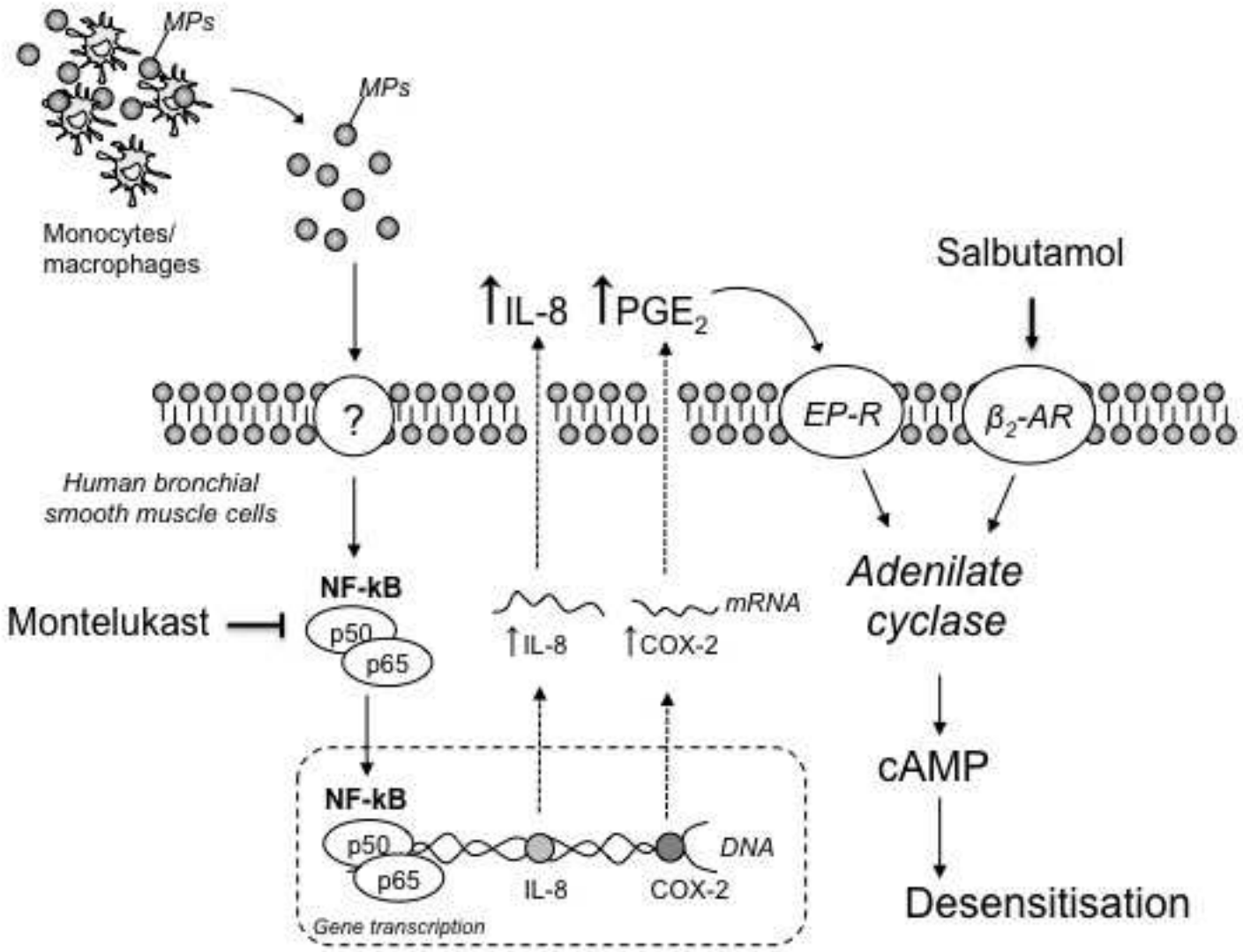

Figure 8

*Graphical Abstract (for review)