



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

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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 desensitization. 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 desensitized 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 desensitization 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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1. Introduction

Microparticles (MPs) are membrane fragments shed by virtually all eukaryotic cells upon activation or during apoptosis that play a significant role in intercellular communication and also in several pathophysiological processes including coagulation and inflammation [1]. Although MPs have been the object of intense experimental research in several pathologic conditions including atherosclerosis, diabetes, sepsis, arthritis, and cancer [2], their contribute to the pathophysiological changes observed in chronic respiratory diseases is currently unknown. In the past few years, our research has been focused on monocyte-derived MPs because 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 [3]. In particular, we demonstrated that human monocyte/macrophage-derived MPs up-regulate inflammatory mediator synthesis by human airway epithelial cells [4,5] and elicit an autocrine activation loop by inducing superoxide anion production, cytokine release and NF-κB activation [6]. 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 [3].

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, at least in an animal model of acute asthma, has emerged for the possible use of high-dose MK as anti-inflammatory agent in acute asthma [7].

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The mechanisms by which MK can elicit these secondary CysLTRs-independent effects have been recently reported [8] 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. The local Review Board approved the protocol and all subjects signed an informed written consent. MPs generation was obtained from adherent cells stimulated with the calcium ionophore, A23187, as previously described [5]. Briefly, human monocytes (about 2×10^6 cells/well) were stimulated by the calcium ionophore A23187 at $12 \mu\text{M}$ in a final volume of 0.5 ml for 20 min at 37°C and the supernatant was recovered and cleared by centrifugation ($14,000 \times g$, 5 min, room temperature).

2.2. Flow cytometry analysis

MPs were characterized by multiparametric flow cytometry analysis (BD FACSCalibur™ Software; Becton Dickinson, San Jose, CA) of the monocyte markers CD14 (the receptor for LPS) and CD16 (the low affinity receptor for IgG), using a blend of fluorescent beads (Sigma–Aldrich, USA) of three diameters (0.1, 0.5 and $1 \mu\text{m}$), 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.

2.3. Human bronchial smooth muscle cells

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 $\mu\text{g}/\text{ml}$ 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). Before experiments, human BSMC were serum-starved for 24 h in serum-free medium to avoid the influence of growth factors on BSMC phenotype.

2.4. 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'-AGGGACAGGTCTTGGTGTG-3' (R), for COX-1; 5'-TGAAACCCACTCCAAACACA-3' (F) and 5'-AACTGATGCGTGAAGTGCTG-3' (R) for COX-2; 5'-ATGACTTCCAAGCTGGCCGT-3' (F) and 5'-CCTCTCAAA-AACTTCTCCACACC-3' (R) for IL-8; 5'-ACAAGGACGCCATCAACTG-3' (F) and 5'-AAAGACCATAACCACCAAGGG-3' (R) for β 2-adrenoceptor; 5'-GTGAAGGTCTGGAGTCAACG-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' software (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. 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.

2.6. cAMP assay

Heterologous β 2-adrenoceptor desensitization was tested by exposing human BSMC to monocyte-derived MPs for 24 h. Intracellular cAMP levels were measured by the cAMP-Glo™ Assay (Promega, Madison, WI, USA) in control and desensitized cells after stimulation with $10 \mu\text{M}$ salbutamol, in the presence or absence of $1 \mu\text{M}$ Bay 11-7082 or $30 \mu\text{M}$ montelukast for 24 h. BSMC were used between passages 4 and 8.

2.7. Electrophoretic mobility shift assay (EMSA)

The activation of NF- κ B induced by MPs was evaluated by measuring its nuclear migration (by electrophoretic mobility shift assay, EMSA), as previously described [5]. Nuclear extracts (5 μg ; obtained by using the Nuclear extract Kit, Active Motif, USA) from BSMC were incubated with 2 μg poly (dI-dC) and [^{32}P]ATP-labeled 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- κ B consensus oligonucleotide (5'-AGTTGAGGGGACTTCCCAGGC-3') was from Promega. The nucleotide-protein complex was separated on a 5% polyacrylamide gel in $0.5 \times$ 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 μg of commercial antibodies (anti-NF- κ B p65: sc-8008 X from Santa Cruz Biotech, USA; anti-NF- κ B p50: ab7549 from Abcam). Densitometric analysis of NF- κ B nuclear translocation was performed and expressed as arbitrary units.

2.8. Drugs

Salbutamol emisulphate, indomethacin, NS398 and Bay 11-7082 were purchased from Sigma–Aldrich (St. Louis, MO, USA). MK sodium was provided from Merck Research Laboratories (Merck & Co., Inc., Rahway, NJ, USA). Drugs were dissolved as recommended by the manufacturers.

2.9. Data analysis and statistics

All experiments were done in triplicate and data were expressed as mean $\pm$ 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 Bonferroni's post hoc test for multiple comparisons or *t*-test for independent samples in the presence of only two groups to compare; $p < 0.05$ was taken as level of significance.

3. Results

3.1. Characterization of monocyte-derived MPs

As reported in Fig. 1, unstimulated monocytes are 98% CD14⁺ (Fig. 1A), whereas MPs generated from A23187-stimulated

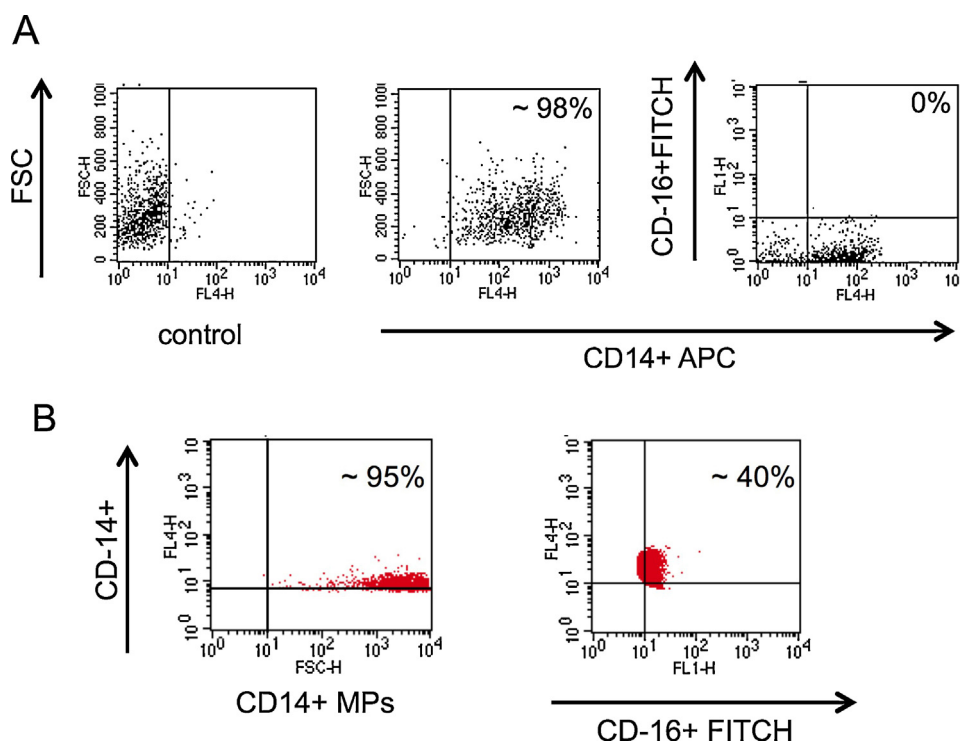


Fig. 1. Flow cytometry analysis of human monocytes and MPs from A23187-challenged monocytes. Figure shows a forward light scatter versus CD14 (the receptor for LPS) and CD16 (the low affinity receptor for IgG) expression dot plot of unstimulated monocytes (A) and MPs generated from A23187-stimulated monocytes (B). This figure is representative of five experiments.

monocytes are 95% CD14⁺ and about 40% CD16⁺ (Fig. 1B), so indicating the pro-inflammatory potential of the ionophore. As known, expression of the marker CD16 distinguishes three monocyte subsets [9]: classical monocytes (CD14⁺⁺CD16[−]), characterized by a poor cytokine release, intermediate monocytes (CD14⁺⁺CD16⁺), non-classical monocytes (CD14⁺CD16⁺⁺). CD16⁺ subpopulations are described to release higher amounts of pro-inflammatory cytokines following stimulation by Toll-like receptor ligands [8]. Results obtained in individual preparations from five different healthy volunteers indicate that unstimulated monocytes belong to the classical monocyte subset ($98 \pm 4\%$ CD14-positive, 0% CD16-positive), while MPs generated from A23187-challenged monocytes present phenotype markers suggestive for the intermediate monocyte subset ($95 \pm 4.8\%$ CD14-positive, $40 \pm 10\%$ CD16-positive; mean $\pm$ SEM).

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 Fig. 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 (Fig. 2A). Noteworthy, such an effect was also accompanied by the release of PGE2 from BSMC into the culture medium (505.6 ± 70.4 pg/ml), about 2.5-fold higher than control ($p < 0.001$; Fig. 2B).

3.3. Effect of MPs on β 2-adrenoceptor function

Following MP stimulation, no effect was observed on the expression of β 2-adrenoceptor gene transcription in BSMC (Fig. 2A). Responsiveness to acute salbutamol at $10 \mu\text{M}$ was assessed in cells exposed to monocyte-derived MPs by measuring intracellular cAMP synthesis. Salbutamol ability to induce cAMP synthesis in BSMC was reduced from 218 ± 25 nM in unstimulated to 108 ± 19 nM after treatment with MPs ($p < 0.01$; Fig. 2C).

3.4. Role of the COX/PGE2 pathway in MP-induced β 2-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 characterize the role of COX enzymes on MP-induced β 2-adrenoceptor desensitization in BSMC. Treatment with indomethacin or NS398 at $1 \mu\text{M}$ for 24 h completely restored salbutamol response in MP-desensitized cells, at levels comparable to those obtained in unstimulated cells (Fig. 3A and B). There is a significant difference between control and indomethacin alone ($p < 0.01$) whereas no change was observed between control and NS398 alone. This might be related to differences in COX selectivity and/or the occurrence of an off-target effect.

In our experimental condition, heterologous β 2-adrenoceptor desensitization was significantly induced, by treating unstimulated BSMC with exogenous PGE2 at a concentration of 100 nM for 24 h, whereas no difference was observed at lower concentrations (Fig. 3C).

3.5. Effect of MPs on the IL-8 expression

The IL-8 mRNA levels were analyzed by RT-PCR after 24 h of vehicle (control) or MPs and compared. A small but significant

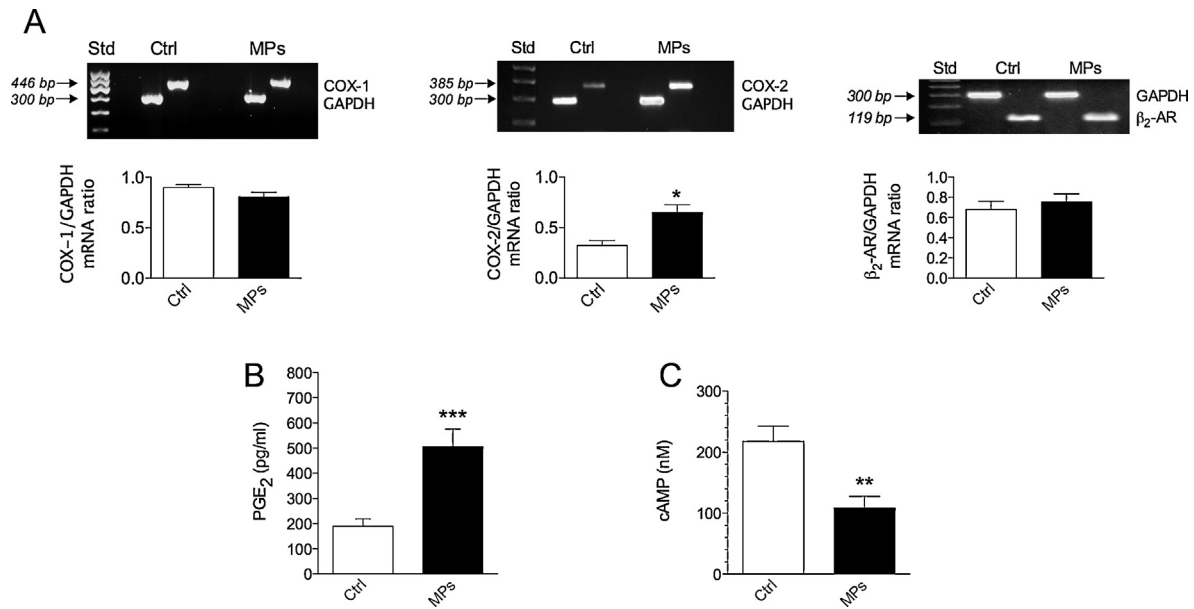


Fig. 2. COX and β₂-adrenoceptor gene expression (A), prostaglandin (PG)-E₂ release (B) and β₂-adrenoceptor function (C) in human BSMC upon overnight incubation with monocyte-derived microparticles (MPs). Control is represented by unstimulated BSMC. Data are reported as mean ± standard error of the mean (SEM). **p* < 0.05, ***p* < 0.01, ****p* < 0.001, compared with control group (*t*-test for independent samples; *n* = 5).

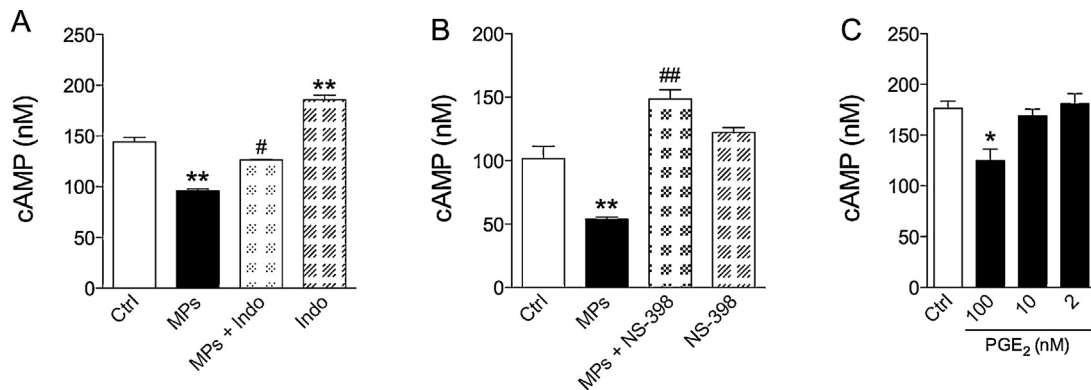


Fig. 3. Effects of indomethacin (non-selective COX inhibitor) (A) and NS398 (selective COX-2 inhibitor) (B) on MP-induced β₂-adrenoceptor desensitization in human BSMC. Heterologous β₂-adrenoceptor desensitization induced by exogenous PGE₂ (C). Control is represented by unstimulated BSMC. Data are reported as mean ± standard error of the mean (SEM). **p* < 0.05, ***p* < 0.01, compared with control group; #*p* < 0.05, ##*p* < 0.01, compared with MP group (ANOVA followed by the Bonferroni's multiple comparison test; *n* = 5).

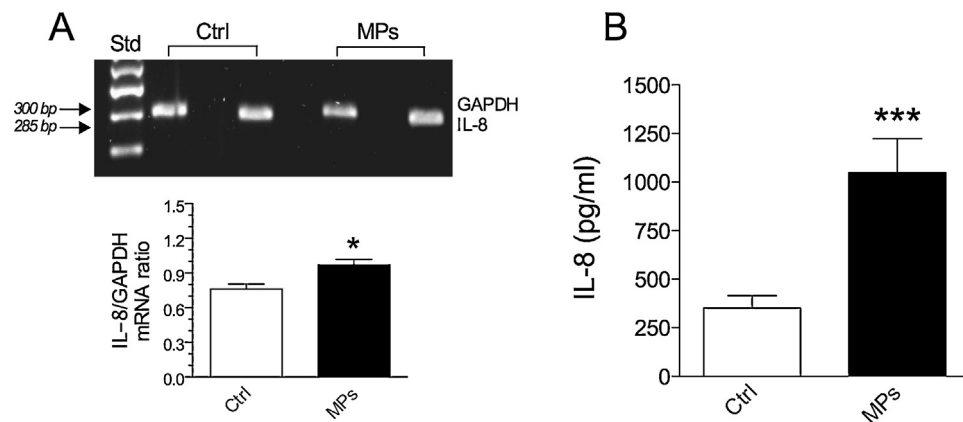


Fig. 4. (A) RT-PCR images and densitometric analyses of interleukin (IL)-8 gene expression and (B) IL-8 release by MP-stimulated human BSMC. Control is represented by unstimulated BSMC. Data are reported as mean ± standard error of the mean (SEM). **p* < 0.05, ****p* < 0.001, compared with control group (*t*-test for independent samples; *n* = 5).

increase in IL-8 mRNA was induced by monocyte-derived MPs (Fig. 4A). Unstimulated BSMC constitutively release an average of 350 ± 66 pg/ml IL-8 and overnight incubation with monocyte-derived MPs caused a 3-fold increase (1048 ± 175 pg/ml; $p < 0.001$) of the IL-8 release into the culture medium (Fig. 4B).

3.6. Role of NF- κ B in MP-induced pro-inflammatory phenotype

The selective inhibitor of NF- κ B, Bay 11-7082, at 1 μ M for 24 h, completely blocked the release of IL-8 (Fig. 5A) and PGE2 (Fig. 5B) in MP-stimulated BSMC without affecting cytokine synthesis in unstimulated cells. Bay 11-7082, at the concentration 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- κ B in BSMC (Fig. 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 Fig. 6).

3.7. Effect of montelukast on MP-induced NF- κ B nuclear translocation

Montelukast 30 μ M reduced translocation of the p50/p65 complex into the nucleus induced by MPs in BSMC, at levels comparable to those of unstimulated cells (Fig. 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 β 2-adrenoceptor desensitization and IL-8 release

Montelukast 30 μ M restored salbutamol response in MP-desensitized cells at levels similar to those of controls, whereas drug alone did not significantly affect cAMP synthesis (Fig. 7A).

Montelukast significantly reduced the IL-8 release ($p < 0.01$) from MP-stimulated cells without affecting the baseline IL-8 levels (Fig. 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 [3,10], the findings of the present study suggest a potential novel mechanism that might contribute to initiate and perpetuate airway inflammation (Fig. 8). Indeed, we demonstrate that human monocyte/macrophage-derived MPs activate important inflammatory pathways in BSMC leading to a switch from a quiescent phenotype to a phenotype expressing a pro-inflammatory secretome. The type of airway smooth muscle response to external stimuli plays an important role in asthma because the secretion of pro-inflammatory mediators by airway smooth muscle cells perpetuates airway inflammation [10]. 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 pro-inflammatory stimuli [11]. 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 [12]. However, it should be noted that dysregulation of the PGE2 synthesis/response axis was also observed in human pulmonary fibrotic

diseases [13] and continuous stimulation by paracrine/autocrine PGE2 promotes heterologous β 2-adrenoceptor desensitization in airway smooth muscle cells [14,15].

The molecular mechanism of β 2-adrenoceptor desensitization induced by cytokines, COX products and contractile agents has been extensively described primarily by the lab of Raymond Penn since the late 90 [16–18] and most recently by other Authors [19,20]. In our experimental setting, prolonged (24 h) in vitro exposure of BSMC to monocyte-derived MPs was carried out to mimic a chronic, inflammatory condition that occurs in patients with chronic asthma. Noteworthy, using inhibitors of cyclooxygenase, we found that β 2-adrenoceptor desensitization was mediated through BSMC derived COX-2 induced prostaglandins. These findings are in line with those showing that indomethacin could prevent interleukin (IL)-1 β -promoted COX-2 induction of PGE2 and β 2-adrenoceptor desensitization in primary human airway smooth muscle cells [21]. However, it is worth mentioning that monocyte-derived MPs might induce the release of various prostaglandin isotypes other than just PGE2. Therefore it is also possible that induction of COX-2 expression by MPs may promote a release of a pool of potential candidates in human BSMC that, alone or in combination, may cause β 2-adrenoceptor desensitization. According to this, it was found that autocrine PGF_{2a}, PGE2 and MRE-269 production caused β 2-adrenoceptor desensitization in rhinovirus infected bronchial epithelial cells [20]. Overall, our findings suggest that activation of the COX-2/prostanoid pathway by human monocyte/macrophages-derived MPs might promote bronchomotor tone dysregulation and altered response to β 2-adrenoceptor agonists (Fig. 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). However, since also lipoxygenase-derived products may account, at least partially, for the heterologous β 2-adrenoceptor desensitization observed in airway smooth muscle cells [22], we cannot exclude a possible role of leukotrienes in the biological effects induced by monocyte-derived MPs.

Elevated levels of IL-8 were found in the airway of patients with cystic fibrosis and asthma [23]. Furthermore, IL-8 works as a potent chemo-attractant for neutrophil that is the primary effector cell in COPD and severe asthma [3], and there are evidence establishing novel roles for IL-8 in human airway smooth muscle cell contraction and proliferation [23,24]. 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 (Fig. 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- κ B pathway plays an important role in the TNF- α -induced pro-inflammatory phenotype in human airway smooth muscle cells [25]. We clearly showed that activation of the nuclear NF- κ B pathway was the common molecular mechanism leading to the up-regulation of PGE2 and IL-8 in MP-stimulated BSMC (Fig. 8). In particular, after exposure of human BSMC to MPs, the released p50/p65 complex translocated into the nucleus and activated the transcription of NF- κ B-regulated genes, including those coding for COX-2 and IL-8. Enhanced nuclear translocation of NF- κ B 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 [5] and in human monocytes [6] 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 prevented nuclear translocation

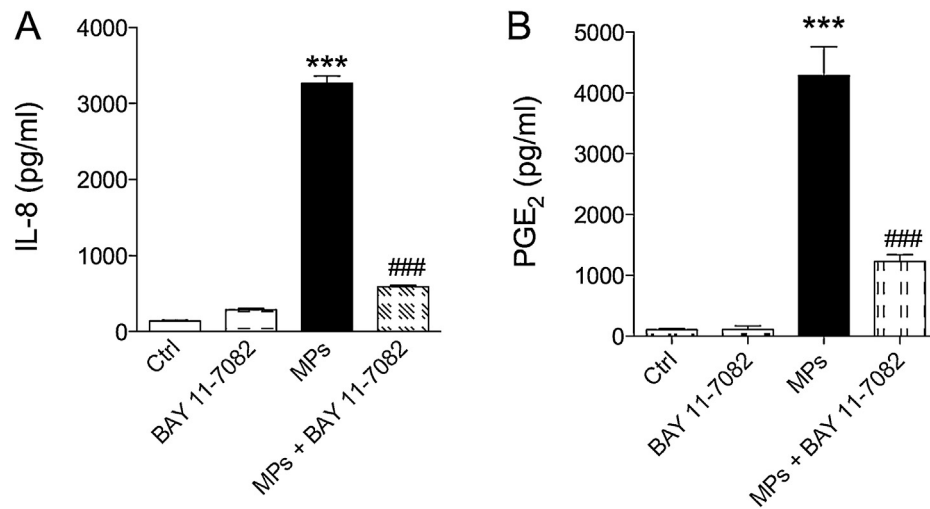


Fig. 5. Effects of the selective inhibitor of NF- κ B, Bay 11-7082, at 1 μ M for 24 h, on the release of IL-8 (A) and PGE₂ (B) by MP-stimulated human BSMC. Control is represented by unstimulated BSMC. Data are reported as mean $\pm$ standard error of the mean (SEM). *** p < 0.001, compared with control group; ### p < 0.001, compared with MP group (ANOVA followed by the Bonferroni's multiple comparison test; n = 5).

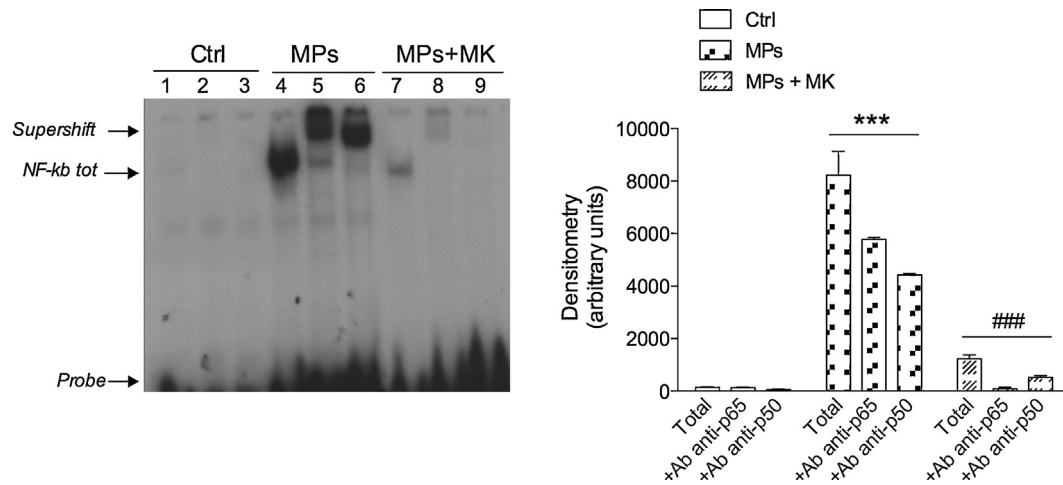


Fig. 6. Image and densitometric analysis of nuclear factor (NF)- κ B activation in human BSMC stimulated with MPs in the presence or absence of montelukast 30 μ M. Lanes 1–3: control, unstimulated BSMC; lanes 4–6: MP-challenged BSMC; lanes 7–9: montelukast (MK) + MPs. Specific antibodies against p65 (lanes 2, 5, 8) and p50 subunits (lanes 3, 6, 9) were used to confirm the specificity of the assay (see text for further details). Control is represented by unstimulated BSMC. Data are reported as mean $\pm$ standard error of the mean (SEM). *** p < 0.001, compared with control group; ### p < 0.001, compared with MP group (ANOVA followed by the Bonferroni's multiple comparison test; n = 3).

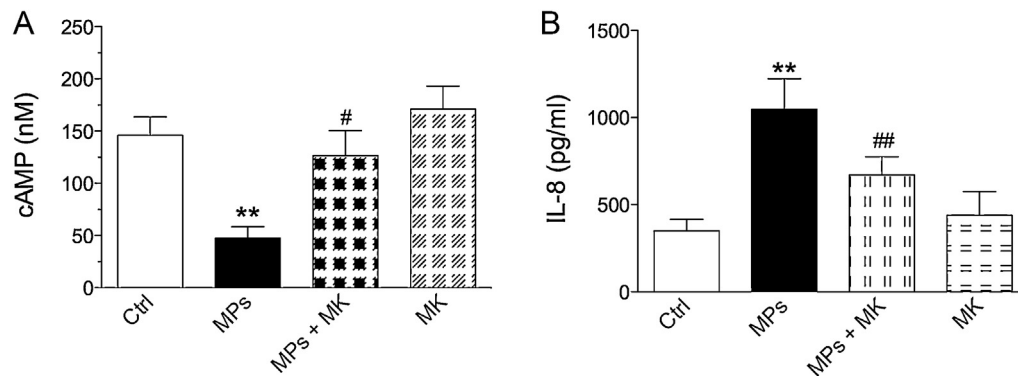


Fig. 7. Effect of montelukast (MK) on MP-induced β 2-adrenoceptor desensitization (A) and IL-8 release (B) in human BSMC. Control is represented by unstimulated BSMC. Data are reported as mean $\pm$ standard error of the mean (SEM). ** p < 0.01, compared with control group; # p < 0.05, ## p < 0.01, compared with MP group (ANOVA followed by the Bonferroni's multiple comparison test; n = 5).

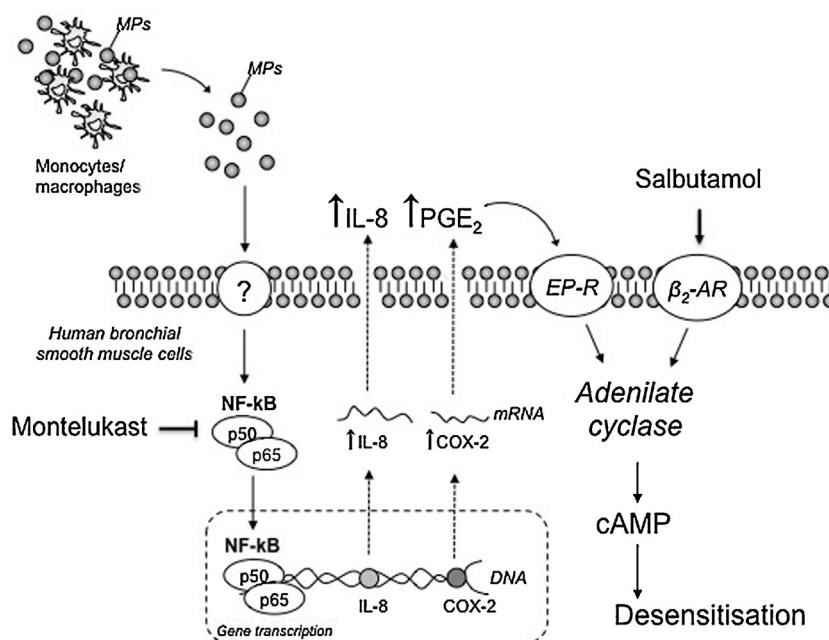


Fig. 8. Overview of the possible molecular mechanisms involved in the pro-inflammatory switch induced by monocyte-derived MPs in human bronchial smooth muscle cells (BSC) and the protective effect of montelukast in this specific setting.

of NF-κB, blocked IL-8 and PGE₂ release and restored salbutamol response in stimulated BSC (Fig. 8). Such an effect was similar to that obtained in our experimental conditions with Bay 11-7082, a well-recognized selective NF-κB inhibitor commonly used as a pharmacological tool to test the role of this signaling pathway in different cell types, including human airway smooth muscle cells [26,27]. Although the molecular mechanism underlying the inhibitory effect of CysLT₁ receptor antagonists on NF-κB activation remains unclear, several lines of evidence indicate that it is not mediated by antagonism of the CysLT₁ receptor [28–31]. Therefore, together with the recognized 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 [1].

MPs research and clinical analysis may be improved by using highly standardized protocols for MPs 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 standardized 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 [4,6]. In this study and in previously published works [5,6], 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 [4]. 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 10 μg protein concentration (that ensured maximal effects) and to 2 nM equivalents of PS, in agreement with previously published works [6,32,33].

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 BSC 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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