Effects of formoterol and salmeterol on cytokine release from monocyte-derived macrophages

L.E. Donnelly, S.J. Tudhope, P.S. Fenwick and P.J. Barnes

ABSTRACT: Pulmonary macrophages are a target for inhaled therapies. Combinations of long-acting β_2-agonists (LABA) and glucocorticosteroids have been developed for asthma and chronic obstructive pulmonary disease (COPD). This study examined two LABA, salmeterol and formoterol, and the glucocorticosteroid, budesonide, on cytokine release from monocyte-derived macrophages (MDM) to determine whether anti-inflammatory effects observed in patients are due to inhibition of macrophages.

MDM were incubated in the absence or presence of LABA or budesonide prior to stimulation with lipopolysaccharide (LPS). Tumour necrosis factor (TNF)-α, granulocyte macrophage-colony stimulating factor (GM-CSF) and CXC chemokine ligand (CXCL)8 were measured by ELISA.

Formoterol and salmeterol inhibited LPS-stimulated release of TNF-α (mean effective concentration (EC_{50}) 2.4 ± 1.8 and 3.5 ± 2.7 nM, respectively; n=11–16), GM-CSF (EC_{50} 24.6 ± 2.1 and 52.4 ± 40.8 nM, respectively, n=11–12) but not CXCL8 from LPS-stimulated MDM. Budesonide inhibited release of all three cytokines (EC_{50} TNF-α: 1.2 ± 0.4 nM; GM-CSF: 0.4 ± 0.2 nM; CXCL8: 0.4 ± 0.1 nM; n=3–4). Formoterol but not salmeterol elevated cAMP in these cells. These effects were attenuated by β-adrenoceptor antagonists, propranolol and ICI118551. Salmeterol (10^{-7} M) also inhibited formoterol-induced cAMP and formoterol-mediated attenuation of cytokine release. Combining budesonide (0.3 nM) with formoterol, inhibited TNF-α release additively.

LABA may inhibit inflammatory cytokine release from macrophages in a cAMP-independent manner and act additively with budesonide.

KEYWORDS: Budesonide, inflammation, long-acting β_2-agonists, macrophage, tumour necrosis factor-α

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Macrophages are the predominant leukocytes in the lung and have a role in homeostasis [1, 2]. Alveolar macrophages are the sentinel cells of the airways, patrolling the lung surface and scavenging any inhaled particles and pathogens and removing apoptotic cells and other debris. This macrophage function suggests that these cells would be prime targets for inhaled pharmaceutical agents. Current inhaled pharmacotherapies include bronchodilators, such as β_2-agonists, and anti-inflammatory agents, such as glucocorticosteroids. Another role of the macrophage is to respond to the local environment to produce a number of mediators including cytokines and chemokines. Macrophages respond to a number of pro-inflammatory stimuli, including lipopolysaccharide (LPS)-producing inflammatory mediators including tumour necrosis factor (TNF)-α, granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL) CXCL8 [3]. Therefore, these cells have the potential to drive the chronic inflammation observed in a number of lung diseases.

Lung macrophage numbers are increased by more than 20-fold in chronic obstructive pulmonary disease (COPD) and could account for many of the pathophysiological features of COPD [4]. The inflammatory response in COPD is associated with increased expression of inflammatory cytokines. Indeed, levels of TNF-α and CXCL8 are elevated in the sputum of patients with COPD compared with control subjects [5–7]. Similarly, GM-CSF levels are raised in the bronchoalveolar lavage of patients with chronic bronchitis compared with controls and are elevated during exacerbations [8, 9]. The precise source of these inflammatory cytokines is

unknown; however, it is possible that macrophages might be an important source of these cytokines in disease [10]. Although alveolar macrophage numbers are not as pronounced in asthma, they also have the capacity to contribute to the increased inflammatory cytokine load observed in the lungs of these patients. In particular, alveolar macrophages from asthmatic subjects release IL-1β and IL-6 [11], which, in turn, stimulate cluster of differentiation (CD)4+ T-cells to produce IL-5 and hence may contribute to the eosinophilic load associated with asthma.

β2-Agonists are administered to patients primarily for their bronchodilatory effects; however, they may also impact upon inflammation since eicosanoids and IL-1β release from stimulated human peripheral blood monocytes can be inhibited by β2-agonists [12]. However, the anti-inflammatory effects of these drugs on macrophages are less clear. Neither the short-acting β2-agonists, salbutamol or terbutaline, nor the long-acting β2-agonists (LABA), salmeterol or formoterol, inhibit LPS-stimulated IL-β release from alveolar macrophages [13]. Similarly, neither salmeterol nor formoterol have any effect on of LPS-stimulated release of C-C motif chemokine 22 (macrophage-derived chemokine) or CXCL10 (interferon-γ-inducible protein of 10 kDa) from differentiated THP-1 cells [14]. However, salmeterol inhibits thromboxane release from human alveolar macrophages [15]. As cAMP-elevating agents, including phosphodiesterase inhibitors, show anti-inflammatory effects in monocytes and macrophages [16, 17], it is possible that β2-agonists would have similar effects.

Although glucocorticosteroids are effective anti-inflammatory treatments for asthma, they are considerably less effective in reducing the underlying inflammation associated with COPD [18]. Recently, combinations of LABA and glucocorticosteroids have been shown to slow the rate of decline of lung function in COPD patients above the effects of each of the components alone [19, 20]. Combinations of glucocorticosteroids and β2-agonists have been shown to act both synergistically and additively. For example, β2-agonists increase nuclear localisation of glucocorticoid receptors (GR) and subsequent DNA binding in primary human lung fibroblasts, resulting in increased steroid-dependent gene transcription [21]. Salmeterol also enhances glucocorticosteroid-dependent inhibition of allergen-induced cytokine release from peripheral blood mononuclear cells [22] and TNF-α-induced expression of intercellular adhesion molecule 1 in fibroblasts [23]. In addition, low concentrations of formoterol increase the suppressive effect of budesonide on the release of TNF-α-induced GM-CSF from cultured human airway epithelial cells [24].

As the macrophage is very likely to interact with inhaled drugs that have the potential to regulate inflammatory cytokine production from these cells, we have examined the efficacy of two LABA, formoterol and salmeterol, on the release of TNF-α, GM-CSF and CXCL8 from LPS-stimulated monocyte-derived macrophages (MDM). LPS-stimulation was used as a surrogate for gram-negative bacterial stimulus. The lower respiratory tract of many patients with COPD is colonised with gram-negative bacteria [25] and hence is relevant stimulus for this study. MDM differentiated in the presence of GM-CSF are phenotypically similar to alveolar macrophages [26] and we have shown they respond similarly to human lung macrophages [3].

In addition, we have compared the responses of these agents with those of the glucocorticosteroid, budesonide, to ascertain whether inhibiting macrophages may contribute to the efficacy of these drugs in the patient.

MATERIALS AND METHODS

Materials
All reagents were obtained from Sigma (Poole, Dorset, UK), unless otherwise stated. Budesonide and formoterol were kind gifts from AstraZeneca (Lund, Sweden) and prepared in DMSO at a stock concentration of 10 mM; the maximum concentration of DMSO in the cell treatments was 0.1% (volume/volume). Salmeterol was prepared as a 50 mM stock in methanol; the maximum concentration of methanol in the cell treatments was 0.002% (v/v).

Culture of MDM
Whole blood was collected from healthy volunteers from the National Heart and Lung Institute (Imperial College London, London, UK). All subjects were nonsmokers, had normal lung function with no history of lung disease. This study was approved by the Imperial College Research Ethics Committee. Blood was collected in 2 mg·mL^{-1} EDTA and peripheral blood mononuclear cells (PBMC) isolated following dextran (6% (weight/volume)) sedimentation and separation using a discontinuous Percoll gradient as previously described [27]. Cells were resuspended in RPMI 1640 medium supplemented with 2 mM L-glutamine 10,000 U·mL^{-1} penicillin, 10 mg·mL^{-1} streptomycin and 10% (v/v) fetal bovine serum (Invitrogen, Paisley, UK) and seeded into 24-well tissue culture plates at a density of 1×10^6 cells·well^{-1}. Monocytes were isolated from the PBMC fraction by adherence on to cell culture plates for 1 h. After this time, the nonadherent cells were removed by aspiration and media replaced with RPMI 1640 medium supplemented with 2 mM L-glutamine, 10,000 U·mL^{-1} penicillin, 10 mg·mL^{-1} streptomycin, 10% (v/v) fetal bovine serum and 2 ng·mL^{-1} GM-CSF. Cells were cultured for 12 days in this differentiation media with media changes on day 4 and day 7. Cells were considered MDM on day 12.

Cytokine measurements
After 12 days of differentiation, cells were washed with Hank's balanced salt solution (HBSS) and media containing the drug under investigation (either β2-agonists or budesonide) prior to stimulation with 10 ng·mL^{-1} LPS (from *Salmonella enteritidis*) for 20 h. The drug remained in the cell culture media throughout the stimulation period. After this time, cytokines were measured by ELISA. CXCL8, TNF-α and GM-CSF were measured using matched pair antibodies according to the manufacturer's instructions (R&D Systems Europe, Abingdon, UK). The detection limit of the CXCL8 and IL-6 assays were 31 pg·mL^{-1}, the TNF-α and GM-CSF assays were 15.5 pg·mL^{-1} and the IL-1β assay 3.15 pg·mL^{-1}.

Cell viability assay
Cell viability was determined colourimetrically by measuring the reduction of the tetrazolium salt, MTT, to formazan by mitochrondial dehydrogenases. Cells treated with vehicle were considered the control and assigned 100% viability. None of the conditions used in this study altered cell viability significantly (data not shown).
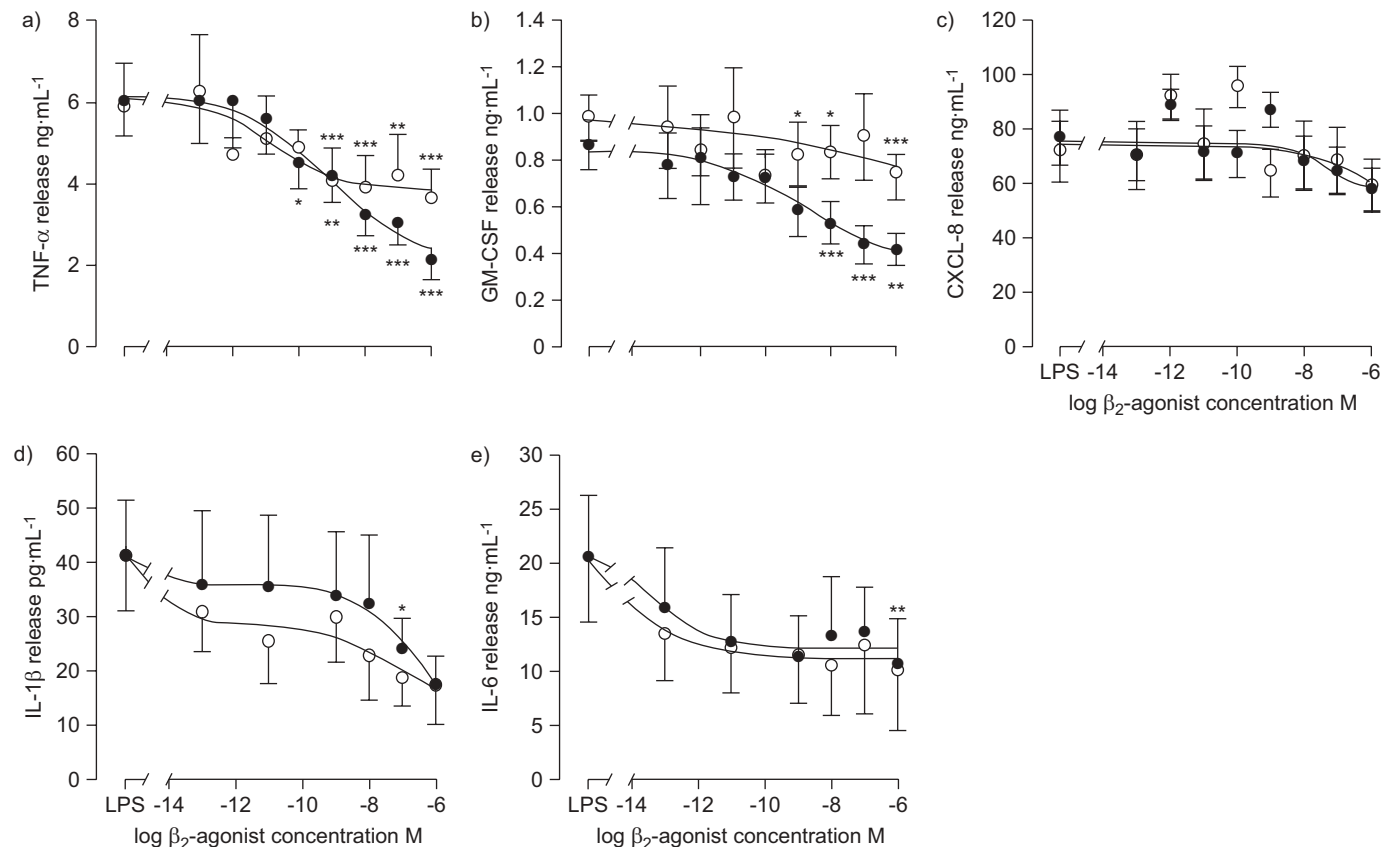
FIGURE 1. Effect of formoterol and salmeterol on lipopolysaccharide (LPS)-stimulated cytokine release from monocyte-derived macrophages (MDM). MDM were pre-treated for 1 h with either formoterol (●) or salmeterol (○) prior to stimulation with 10 ng·mL⁻¹ LPS for 20 h. Cell media were harvested and concentrations of a) tumour necrosis factor (TNF)-α, b) granulocyte macrophage-colony stimulating factor (GM-CSF), c) CXC chemokine ligand (CXCL)8, d) interleukin (IL)-1β, and e) IL-6, were determined using ELISA. Data are presented as mean±SEM for n=5–16. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$ for differences from LPS stimulation.

Measurement of cAMP

MDM were cultured in 24-well plates and incubated in modified HBSS buffer, containing calcium and magnesium, with 0.5% (w/v) bovine serum albumin (BSA). The cells were pre-incubated for 1 h with buffer containing 100 µM 3-isobutyl-1-methylxanthine (IBMX), prior to the addition of β_2-agonists and IBMX remained in the media throughout the experiment. Formoterol (10^{-13}–10^{-6} M) and salmeterol (10^{-13}–10^{-6} M) were incubated with cells for a further 2 mins. Cells were then lysed with 0.1 M HCl containing 0.5% (v/v) tritonX-100. Prostaglandin (PG)E$_2$ at 10^{-6} M was used as a positive control. Samples were stored at -80°C until required. The levels of cAMP in the samples were measured using a commercially available enzyme immunoassay kit, according to the manufacturer's instructions (Biomol International UK, Exeter, UK). The lower limit of detection of the assay was 0.781 pmol·mL⁻¹ cAMP.

Statistics

Data are presented as mean±SEM for n donors. Concentration-response curves were analysed by least-squares, non-linear iterative regression using the ''GraphPad Prism'' curve fitting program (GraphPad software, San Diego, CA, USA) and mean effective concentration (EC50) values were subsequently inter-polated from curves of best-fit. Statistical differences were determined using the Kruskal–Wallis test followed by Dunn's multiple comparison test or a Mann–Whitney test as appropriate. Values of $p < 0.05$ were considered significant. Estimates of antagonist affinity (pK_B) were calculated as described by SCHILD [28] using the equation $pK_B = \log (CR-1)$-log [B], where CR is the concentration ratio calculated as EC50 of agonist in the presence of antagonist divided by the EC50 of the agonist alone and [B] is the concentration of the antagonist [29].

RESULTS

MDM were stimulated with a sub-maximal concentration of LPS (10 ng·mL⁻¹) and the effect of the β_2-agonists, formoterol and salmeterol, on the release of TNF-α, GM-CSF and CXCL8 was evaluated. Formoterol and salmeterol inhibited LPS-stimulated TNF-α release from MDM with EC50 values of 2.4±1.8 nM (n=16) and 3.5±2.7 nM (n=11); with a significantly greater inhibition with formoterol than salmeterol at 10^{-6} M ($p < 0.05$) of 66.5±3.9 and 44.8±8%, respectively (fig. 1a) with significant inhibition with formoterol occurring at 0.1 nM and 1 nM for salmeterol. Both β_2-agonists also inhibited the release of GM-CSF from these cells with EC50 values of 24.6±2.1 nM (n=12) for formoterol and 52.4±40.8 nM (n=11) for salmeterol (fig. 1b). Again, formoterol was more effective at inhibiting LPS-stimulated GM-CSF release from MDM than salmeterol (fig. 1b). By contrast,
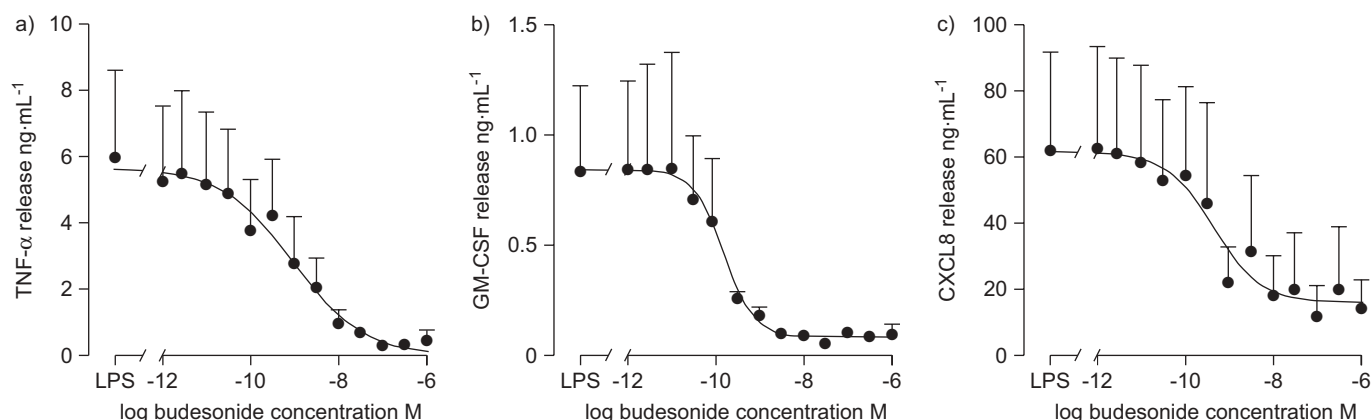
FIGURE 2. Effect of budesonide on lipopolysaccharide (LPS)-stimulated cytokine release from monocyte-derived macrophages (MDM). MDM were pre-treated for 1 h with budesonide prior to stimulation with 10 ng·mL^{-1} LPS for 20 h. Cell media were harvested and concentrations of a) tumour necrosis factor (TNF)-α, b) granulocyte macrophage-colony stimulating factor (GM-CSF), and c) CXC chemokine ligand (CXCL)8, were determined using ELISA. Data are presented as mean$\pm$SEM for n=3–4.

neither formoterol nor salmeterol inhibited the release of CXCL8 from these cells (fig. 1c). Both formoterol and salmeterol inhibited LPS-stimulated IL-1β release from these cells with EC50 values of 110$\pm$84 nM (n=5) and 28$\pm$21 nM (n=5), respectively; and also IL-6 release with EC50 values of 2.5$\pm$2.4 pM (n=5) and 0.04$\pm$0.01 pM (n=5), respectively. The limited inhibitory effects of β_2-agonists on LPS stimulation of MDM was not due to this cellular model being resistant to inhibition since TNF-α, GM-CSF and CXCL8 release from these cells were all inhibited by >80% by the addition of budesonide (fig. 2), with EC50 values of 1.2$\pm$0.4 nM (n=4), 0.4$\pm$0.2 nM (n=4) and 0.4$\pm$0.1 nM (n=3), respectively.

Elevation of intracellular levels of cAMP can lead to inhibition of inflammatory gene transcription; therefore, it was possible that the differential effects of formoterol and salmeterol on the inhibition of cytokine release from LPS-stimulated MDM were due to differences in their capacity to increase cAMP levels in these cells. Experiments were performed in the presence of IBMX (100 μM). Formoterol stimulated accumulation of cAMP in MDM (EC50=1.6$\pm$0.3 nM; n=5) but exposure of the cells to

salmeterol did not alter cAMP levels at any of the concentrations examined (10^{-13}–10^{-6} M) (fig. 3a). Time courses of $\leqslant$15 min were performed but salmeterol failed to elevate cAMP in this system (data not shown). To ascertain that the effect of formoterol was *via* activation of β-receptors, cells were pre-treated with either the nonselective β-adrenoceptor antagonist propranolol (10^{-7} M) or the selective β_2-adrenoceptor antagonist ICI118551 (10^{-7} M) prior to exposure to formoterol. Both antagonists abrogated formoterol-mediated elevation of cAMP (fig. 3b) suggesting that formoterol was acting *via* a β_2-adrenoceptor.

The inability of salmeterol to increase levels of cAMP in MDM prompted investigation as to whether, in these cells; salmeterol could act as an antagonist. To examine this possibility, MDM were pre-treated with 10^{-7} M salmeterol prior to stimulation with formoterol (10^{-13}–10^{-6} M). Pre-treatment of MDM with salmeterol for 30 min did not increase cAMP significantly above baseline levels (baseline: 0.56$\pm$0.14 pmol·mL^{-1} (n=4) *versus* salmeterol: 1.7$\pm$0.4 pmol·mL^{-1} (n=4)). However, salmeterol inhibited formoterol-induced accumulation of cAMP
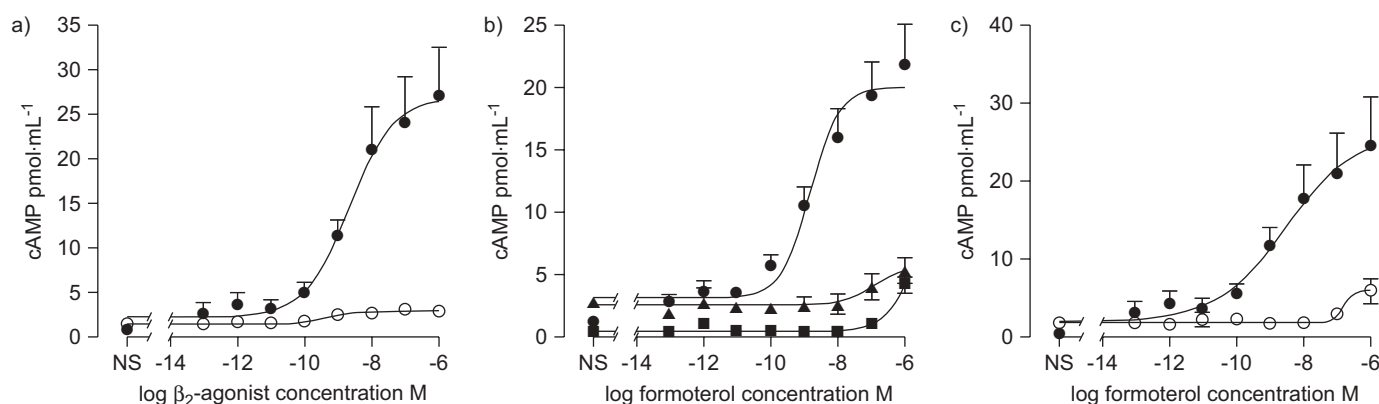

FIGURE 3. Effect of formoterol and salmeterol on elevation of cAMP in monocyte-derived macrophages (MDM). a) MDM were treated with either formoterol ($\bullet$) or salmeterol ($\circ$) for 2 min. Cells were harvested and cAMP levels determined. Data are presented as mean$\pm$SEM for n=5. b) MDM were pre-treated with either 10^{-7} M propranolol ($\blacksquare$) or 10^{-7} M ICI118551 ($\blacktriangle$) for 1 h prior to stimulation with formoterol for 2 min. c) MDM were either pre-treated for 30 min in the absence ($\bullet$) or presence ($\circ$) of 10^{-7} M salmeterol prior to stimulation with formoterol for 2 mins. All experiments were performed in the presence of IBMX (100 μM). Cells were harvested and cAMP levels determined. Data are presented as mean$\pm$SEM for n=3–8.
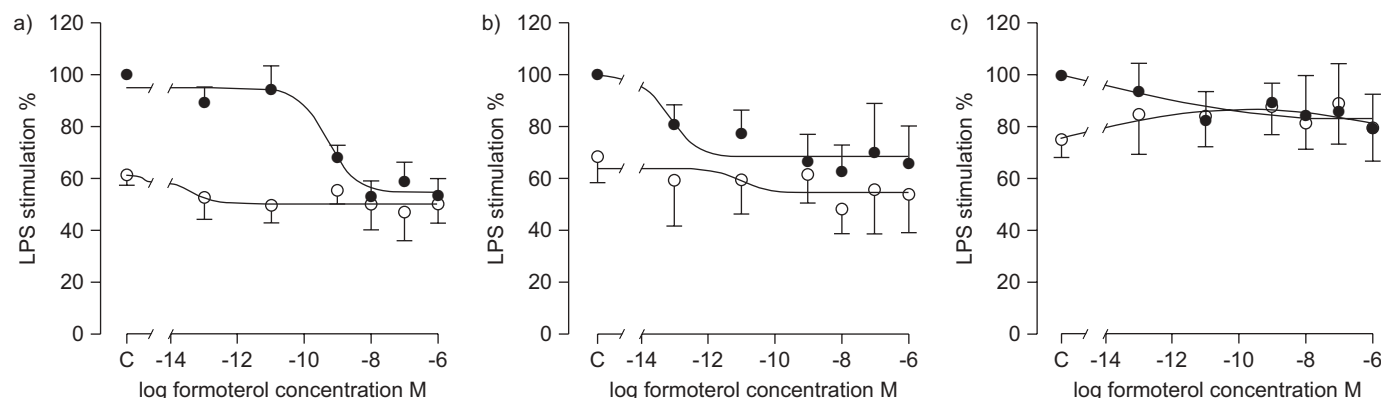
FIGURE 4. Effect of salmeterol on formoterol-mediated inhibition of lipopolysaccharide (LPS)-stimulated cytokine release from monocyte-derived macrophages (MDM). MDM were either pre-treated for 30 min in the absence (●) or presence (○) of 10^{-7} M salmeterol prior to incubation with formoterol for a further 1 h. Cells were stimulated with 10 ng·mL^{-1} LPS for 20 h, media harvested and the concentrations of a) tumour necrosis factor-α, b) granulocyte macrophage-colony stimulating factor, and c) CXC chemokine ligand 8 were determined by ELISA. Data are presented as mean±SEM, n=6, where 100% is the maximal stimulation with LPS. C: control.

in MDM (fig. 3c). These data suggested that salmeterol, which is a partial agonist, could act antagonistically at the β$_2$-receptor. Therefore, the effect of salmeterol to attenuate the inhibitory effects of formoterol on cytokine release was evaluated. MDM were pre-treated with 10^{-7} M salmeterol prior to exposure to formoterol then stimulated with LPS and cytokines measured. This concentration of salmeterol inhibited TNF-α by ~40%, GM-CSF by ~30% and CXCL8 by ~20% (fig. 4). However, despite inhibiting the accumulation of formoterol-stimulated cAMP in MDM (fig. 3c), the addition of formoterol following salmeterol pre-incubation did not result in further inhibition of LPS-stimulated cytokine release (fig. 4). To further investigate the mechanism underlying the differential inhibitory effects of formoterol and salmeterol, MDM were stimulated with LPS for 20 h following 30 min pre-treatment with a phosphodiesterase (PDE) inhibitor, rolipram (10 μM) in the presence of either formoterol or salmeterol and the release of TNF-α measured. Inhibition of PDE enhanced the inhibitory effect of formoterol (fig. 5a) but not salmeterol (fig. 5b). LPS stimulation of these cells was unlikely to be generating other, endogenous agents

(e.g. prostaglandin E$_2$) that could contribute to the inhibitory effects observed, as there was no difference in the level of TNF-α released from these cells following stimulation with LPS in the absence or presence of rolipram (11.9±3.3 versus 12.3±4.4 ng·mL^{-1}; n=4).

The possibility that formoterol-mediated inhibition of LPS-stimulated cytokine release from MDM was not mediated via the β-adrenoceptor was investigated using propranolol and ICI118551. However, both antagonists attenuated the inhibitory action of formoterol on the release of LPS-stimulated TNF-α from MDM (fig. 6) (EC$_{50}$ formoterol 0.17±0.07 nM (n=4); formoterol and propranolol 60.7±36.1 nM (n=3); and formoterol and ICI118551 22.7±22.5 nM (n=4)) with estimated pK_B values of 9.0±0.7 and 8.3±0.7 for propranolol and ICI118551 respectively. This suggested that formoterol inhibits cytokine release via the β$_2$-adrenoceptor on MDM.

It has been reported that steroids and β$_2$-agonists can act either synergistically or additively to inhibit inflammatory cytokines in a variety of cell systems [30, 31]. Therefore, we investigated
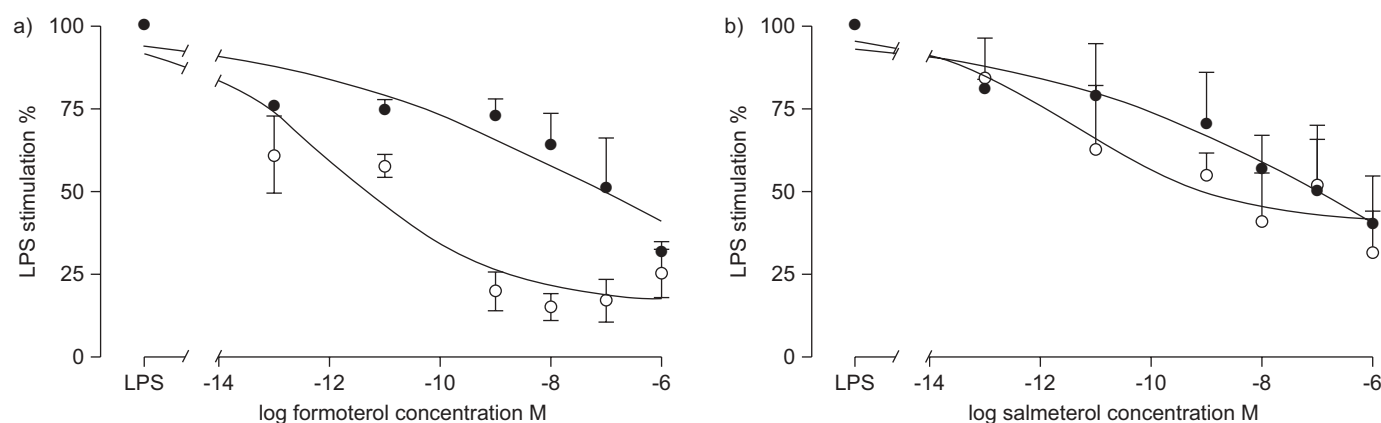

FIGURE 5. Effect of rolipram on formoterol or salmeterol-mediated inhibition of lipopolysaccharide (LPS)-stimulated tumour necrosis factor (TNF)-α release from monocyte-derived macrophages (MDM). MDM were either pre-treated for 30 mins in the absence (●) or presence (○) of 0.3 nM budesonide prior to incubation with a) formoterol or b) salmeterol for a further 1 h. Cells were stimulated with 10 ng·mL^{-1} LPS for 20 h, media harvested and the concentrations of TNF-α were determined by ELISA. Data are presented as mean±SEM, n=4.
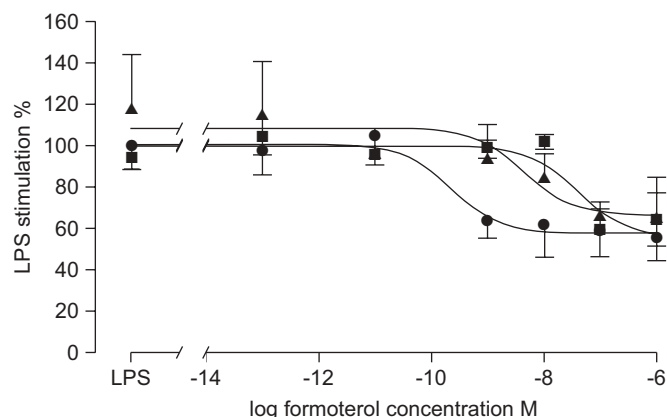
FIGURE 6. Effect of β-adrenoceptor antagonists on formoterol-mediated inhibition of lipopolysaccharide (LPS)-stimulated cytokine release from monocyte-derived macrophages (MDM). MDM were either pre-treated for 30 min in the absence (●) or presence of 10^{-7} M propranolol (■) or 10^{-7} M ICI118551 (▲) prior to incubation with formoterol for a further 1 h. Cells were stimulated with 10 ng·mL^{-1} LPS for 20 h, media harvested and the concentrations of tumour necrosis factor-α were determined by ELISA. Data are presented as mean±SEM, n=3–4, where 100% is the maximal stimulation with LPS.

whether a low concentration of budesonide (0.3 nM) would alter the anti-inflammatory effects of formoterol on LPS-stimulated MDM. Budesonide alone (0.3 nM) inhibited LPS-stimulated TNF-α, GM-CSF and CXCL8 release by 32.3±9.8% (n=5), 61.3±11.2% (n=3) and 28.8±4.3% (n=4), respectively (fig. 7). Formoterol alone inhibited LPS-stimulated TNF-α and GM-CSF release with EC50 values of 0.2±0.1 nM (n=5) and 0.8±0.3 nM (n=3), respectively but there was no effect of formoterol on LPS-stimulated CXCL8 release from MDM. The combination of 0.3 nM budesonide with formoterol inhibited TNF-α, and GM-CSF release with EC50 values for formoterol of 1.1±1.0 nM (n=5) and 1.6±1.5 nM (n=3), respectively (fig. 7a and b) with no significant shifts in the formoterol response curves. The presence of budesonide did

not alter the formoterol response to CXCL8 release from these cells (fig. 7c)

DISCUSSION

Alveolar macrophages will interact with inhaled pharmaceutical agents and, as such, are a good target for therapeutic interventions. Macrophages have been implicated in the pathophysiology of COPD [4] and may contribute to inflammatory load in asthma; therefore, many therapeutic approaches are being developed that aim to target these cells directly. Hence, it is important to understand how current pulmonary medications may interact with macrophages and whether these drugs could regulate the release of inflammatory mediators from these cells. Inflammation in COPD is associated with increased concentrations of cytokines including TNF-α, GM-CSF and CXCL8 [4]; therefore, this study examined the effects of LABA on the release of these mediators from LPS-stimulated macrophages together with IL-1β and IL-6 which may contribute to the pathophysiology of asthma. LPS was selected as a stimulus as it represents the presence of gram-negative bacteria. There are limitations to this approach, as the LPS used may not be of the strain found in the airway; nevertheless, LPS is considered the classical activator of macrophages and was used in this study as a pro-inflammatory stimulus [32].

This study demonstrated that the full β$_2$-agonist, formoterol, was more effective than the partial β$_2$-agonist, salmeterol, at inhibiting TNF-α and GM-CSF release from LPS-stimulated MDM. This concurs with studies examining the effects of these β$_2$-agonists on histamine release from mast cells and mediator release from eosinophils [33] where salmeterol is less efficacious than formoterol [34]. The data presented in this study differ from that observed in THP-1 cells that were differentiated towards a macrophage phenotype with phorbol myristate acetate, where neither formoterol nor salmeterol inhibit LPS-stimulated TNF-α release [14]. In the present study, neither of the β$_2$-agonists examined inhibited LPS-stimulated CXCL8 release from these macrophages. This would suggest that the reported effects of formoterol to reduced sputum
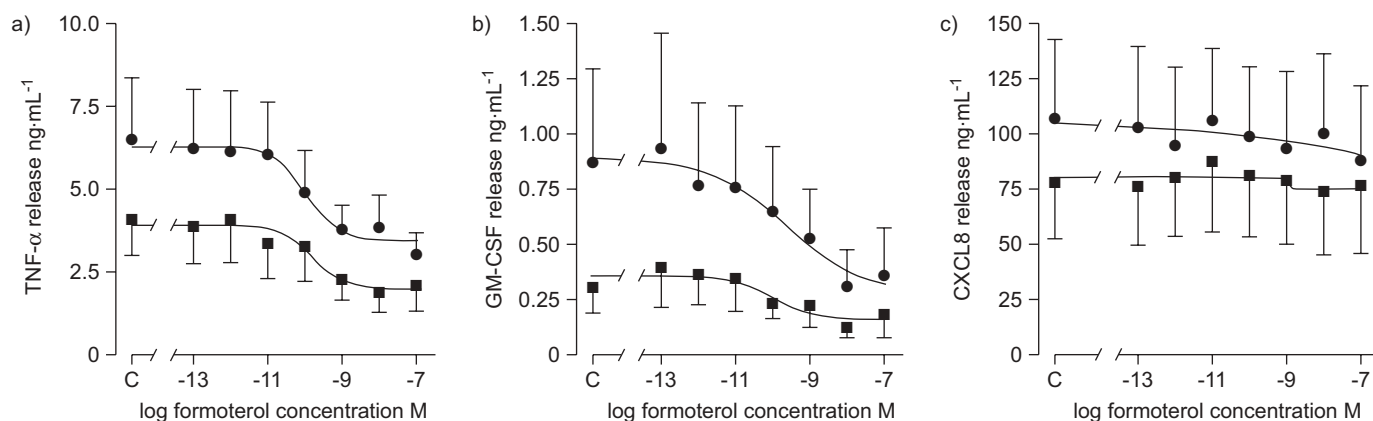

FIGURE 7. Effect of budesonide on formoterol-mediated inhibition of lipopolysaccharide (LPS)-stimulated cytokine release from monocyte-derived macrophages (MDM). MDM were either pre-treated for 30 min in the absence (●) or presence (■) of 0.3 nM budesonide prior to incubation with formoterol for a further 1 h. Cells were stimulated with 10 ng·mL^{-1} LPS for 20 h, media harvested and the concentrations of a) tumour necrosis factor (TNF)-α, b) granulocyte macrophage-colony stimulating factor (GM-CSF), and c) CXC chemokine ligand (CXCL)8 were determined by ELISA. Data are presented as mean±SEM, n=3–5.

CXCL8 [35] and salmeterol to reduce bronchoalveolar lavage fluid CXCL8 in asthma [36] are not due to inhibition of macrophages. There was also inhibitory effect of both LABA on LPS-stimulated release of IL-1β and IL-6. The former contrasts with data presented for alveolar macrophages whereby LABA failed to inhibit LPS-stimulated IL-β release from alveolar macrophages [13] and may highlight differences in this model system. However, the present study demonstrates differential effects of β2-agonists on inflammatory mediator production from macrophages and this is not restricted to pro-inflammatory cytokines, since IL-6 can also be inhibited. The lack of effect of β2-agonists on CXCL8 release has been shown previously in human primary smooth muscle cells stimulated with IL-1β [37]. LPS-stimulation of cytokine release is thought to be mediated *via* the activation of nuclear factor (NF)-κB [38], which can be inhibited by cAMP [39, 40], although we have shown that this is not the case for CXCL8. These data demonstrate that different inflammatory genes regulated by NF-κB are themselves differentially regulated by cAMP-elevating agents.

However, our study suggests that inhibition of inflammatory gene expression *via* β2-agonists may not require elevation of cAMP. Salmeterol failed to elevate cAMP in this system similar to the effect reported in human airway smooth cells [41], yet could inhibit TNF-α and GM-CSF release, albeit to reduced levels compared with formoterol. The lack of a relationship between the inhibitory effect of salmeterol and cAMP elevation was further supported by the observation that inhibition of PDE could not enhance the inhibitory effect of salmeterol but increased formoterol mediated inhibition. Salmeterol also acted as an antagonist to prevent formoterol mediated elevation of cAMP in these cells. This observation that salmeterol can act as an antagonist at the β2-adrenceptor has been reported previously in human eosinophils [42]. Similarly, pre-treatment of MDM with salmeterol blocked any further inhibition of TNF-α release from these cells in the presence of formoterol. The limited effect of salmeterol in this system suggests that β2-adrenoceptor-mediated inhibition of cytokine release from macrophages is, in part, cAMP-independent and requires alternative signalling pathways. Recently, it has been demonstrated that β-adrenoceptors can switch coupling from Gs to Gi [43]; however, since formoterol elevates cAMP in MDM, this is an unlikely explanation in MDM. Alternative β2-adrenoceptor signalling pathways have been proposed [44, 45]. For example, β2-adrenoceptors coupling to Gs can mediate smooth muscle relaxation in a cAMP-independent manner [46, 47]. We have now demonstrated an anti-inflammatory effect of LABA; for example, formoterol is β-adrenoceptor-dependent but salmeterol can mediate a cAMP-independent mechanism in MDM. This may have clinical relevance, since the inhibitory effect of salmeterol against TNF-α, with no concomitant increase in cAMP occurred from 1 nM, which is considered in the therapeutic range.

Recently, there has been growing interest in the mechanism(s) of the enhanced effects of combinations of LABA and glucocorticosteroids [48, 31]. In order to investigate whether macrophages could account for the enhanced anti-inflammatory effects of combinations of LABA and steroids, we examined the effect of a low dose (0.3 nM) budesonide on the anti-inflammatory actions of formoterol. It has been proposed

that LABA act synergistically with steroids by a number of mechanisms. These include enhanced translocation of the GR to the nucleus [21, 49] leading to increase GR-dependent transcription [50]. One gene that is upregulated in this manner is mitogen-activated protein (MAP) kinase phosphatase-1, an inhibitor of MAP kinase signalling pathways [51]. Inhibition of MAP kinase signalling in LPS-stimulated MDM leads to attenuation of cytokine release [3] and could account for any synergistic effects of LABA and glucocorticosteroids. However, in this study, we observed additive effects of budesonide and formoterol indicating that inhibition of LPS-stimulated cytokine release is likely to occur *via* separate pathways in MDM. This suggests that macrophages are unlikely to account for any synergistic anti-inflammatory effects of LABA and glucocorticosteroids observed in clinical studies.

In summary, formoterol inhibited cytokine release from MDM *via* interaction with the β2-adrenoceptor. Similarly, the partial agonist, salmeterol had a limited anti-inflammatory activity in this cell system but did not stimulate intracellular cAMP levels. Taken together, these data suggest alternative activation pathways for β2-agonists in macrophages that may contribute to the anti-inflammatory actions of these therapeutic agents.

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