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Suppression of granulocyte/macrophage colony-stimulating factor release from human monocytes by cyclic AMP-elevating drugs: role of interleukin-10

[1,2]Paul M. Seldon & *,[1]Mark A. Giembycz

[1]Thoracic Medicine, National Heart and Lung Institute, Imperial College School of Medicine, London SW3 6LY

1 Granulocyte/macrophage colony-stimulating factor (GM-CSF) is a pro-inflammatory cytokine secreted by cells of the monocyte/macrophage lineage and has been implicated in the pathogenesis of bronchitis and asthma.

2 In the present study we have evaluated the effect of several cyclic AMP-elevating agents on lipopolysaccharide (LPS)-induced GM-CSF release from human monocytes and the extent to which the anti-inflammatory cytokine, interleukin (IL)-10, is involved.

3 LPS evoked a concentration-dependent generation of GM-CSF from human monocytes that was inhibited, at the mRNA and protein level, by 8-Br-cyclic AMP, cholera toxin, prostaglandin E_2 (PGE_2) and a number of structurally dissimilar phosphodiesterase (PDE) 4 inhibitors.

4 Pre-treatment of monocytes with a concentration of an anti-IL-10 monoclonal antibody that abolished the inhibitory action of a maximally effective concentration of exogenous human recombinant IL-10, significantly augmented LPS-induced GM-CSF generation. This effect was associated with a parallel upwards displacement of the concentration-response curves that described the inhibition of GM-CSF by PGE_2, 8-Br-cyclic AMP and the PDE4 inhibitor, rolipram, without significantly changing the potency of any drug. Consequently, the maximum percentage inhibition of GM-CSF release was reduced. Further experiments established that the reduction in the maximum inhibition of GM-CSF release seen in anti-IL-10-treated cells was not due to functional antagonism as rolipram, PGE_2 and 8-Br-cyclic AMP were equi-effective at all concentrations of LPS studied.

5 These data indicate that cyclic AMP-elevating drugs attenuate the elaboration of GM-CSF from LPS-stimulated human monocytes by a mechanism that is not mediated *via* IL-10. Suppression of GM-CSF from monocytes may explain, at least in part, the efficacy of PDE4 inhibitors in clinical trials of chronic obstructive pulmonary disease.

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Abbreviations: BAL, bronchoalveolar lavage; 8-Br-cyclic AMP, 8-bromo-cyclic adenosine-3′,5′-monophosphate; cyclic AMP, cyclic adenosine-3′,5′-monophosphate; CTX, cholera toxin; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte/macrophage colony-stimulating factor; HBSS, Hanks' balanced salt solution; IL, interleukin; LPS, lipopolysaccharide; PDE, phosphodiesterase; PGE_2, prostaglandin E_2; TNFα, tumour necrosis factor-α

Introduction

Granulocyte/macrophage colony-stimulating factor (GM-CSF) is a 14–35 kDa acidic glycoprotein that is secreted by many cells including those of the human monocyte/macrophage lineage (Blanchard *et al*., 1991; Meja *et al*., 2000; Sallerfors & Olofsson, 1992). Many studies, *in vitro* and *in vivo*, have shown that GM-CSF primes and activates a wide variety of inflammatory and immune cells and is implicated in the pathogenesis of a number of respiratory diseases including chronic obstructive pulmonary disease (COPD) and asthma. For example, increased expression of GM-CSF has been detected in the bronchoalveolar lavage (BAL) fluid of patients with asthma and in bronchitic subjects during exacerbations (Balbi *et al*., 1997). In addition, the number of $GM\text{-}CSF^+$ T-lymphocytes, eosinophils and monocytes in the BAL fluid of asthmatic subjects after allergen provocation is increased when compared to normal individuals (Broide *et al*., 1991; 1992; Davies *et al*., 1995; Hallsworth *et al*., 1994; Sousa *et al*., 1993; Woolley *et al*., 1995), which is consistent with the elevated level of circulating GM-CSF in people with acute severe asthma (Brown *et al*., 1991). Furthermore, intrapulmonary transfer of the GM-CSF gene to rats leads to eosinophilia and monocytosis that is associated with irreversible fibrosis and airway remodelling (Xing *et al*., 1996). In mice, transfer of the same gene contributes to airways inflammation by prolonging interleukin (IL)-4- and IL-5-driven leukocyte infiltration (Lei *et al*., 1998).

*Author for correspondence at: Thoracic Medicine, National Heart and Lung Institute, Imperial College School of Medicine, Dovehouse Street, London SW3 6LY; E-mail: m.giembycz@ic.ac.uk

[2]Current address: Department of Dermatology, Imperial College School of Medicine, Hammersmith Hospital, Du Cane Road, London W12 0NN

The possible contribution of GM-CSF to the pathogenesis of airway inflammatory diseases provides a compelling rationale for targeting the generation and/or release of this cytokine with small molecule inhibitors. Novel compounds that show promise in the treatment of asthma and COPD include inhibitors of the cyclic AMP-specific, or type 4, family of cyclic nucleotide phosphodiesterases (PDE), some of which are in phase III clinical trials (Giembycz, 2000; 2001; Norman, 2000; Torphy, *et al*., 1999). However, although cyclic AMP-elevating agents generally suppress the production of pro-inflammatory cytokines there is good evidence that cytokine gene expression can also be up-regulated (Zidek, 1999). Indeed, the release of GM-CSF from human bone marrow stromal cells and T-lymphocytes can be attenuated and augmented under certain conditions (Borger *et al*., 1996; Bug *et al*., 1998; Kambayashi *et al*., 1995; Quill *et al*., 1989).

It has been proposed that the ability of PDE4 inhibitors and PGE_2 to suppress the generation of pro-inflammatory cytokines (e.g. tumour necrosis factor-α [TNFα], IL-6) from murine macrophages requires the formation of IL-10 (Kambayashi *et al*., 1995; Strassmann *et al*., 1994). A similar conclusion was reached from *in vivo* studies of endotoxin shock with cell permeant cyclic AMP analogues (Arai *et al*., 1995), iloprost (Grundmann *et al*., 1992), pentoxifylline and a novel alkylxanthine PDE inhibitor, A802715 (Jilg *et al*., 1996). In the former investigation an anti-IL-10 neutralizing antibody inhibited the ability of dibutyryl cyclic AMP to protect against endotoxin-induced liver injury in mice rendered hyper-sensitive to LPS by the intravenous administration of *Propionibacterium acnes*. Collectively, these results led to a proposal that cyclic AMP augments IL-10 secretion in LPS-sensitive cells, which acts in an autocrine manner to suppress the elaboration of pro-inflammatory cytokines (Arai *et al*., 1995; Jilg *et al*., 1996; Kambayashi *et al*., 1995). These results are potentially important given that IL-10 is known to suppress certain Th1- and Th2-driven indices of inflammation (Pretolani & Goldman, 1997; Schreiber *et al*., 1995). Moreover, from a molecular perspective these data are consistent with the description of a cyclic AMP-response element (CRE) within the promoter region of the murine IL-10 gene (Kim *et al*., 1992), and the fact that cyclic AMP-elevating agents increase the number of IL-10 mRNA transcripts and augment IL-10 release (Kambayashi *et al*., 1995).

In the present study, we have evaluated the effect of a variety of cyclic AMP-elevating agents on LPS-induced GM-CSF release from human monocytes and, as the human IL-10 gene also features a putative CRE (Platzer *et al*., 1994; 1995), the extent to which IL-10 is involved.

Methods

Isolation and purification of human mononuclear cells

Blood was collected from normal healthy individuals by antecubital venepuncture into acid citrate dextrose (in mM): disodium citrate 160, glucose 110 – pH 7.4 and mixed with 6% Hespan (hydroxymethyl starch) to sediment erythrocytes. After standing at room temperature for 90 min, the leukocyte-rich plasma was removed and centrifuged at $312 \times g$ for 7 min. The resulting cell pellet was resuspended gently in approximately 7 ml of buffer A (in mM: KH_2PO_4 5, K_2HPO_4 5, NaCl 110 – pH 7.4) made 50% with Percoll and layered over a discontinuous Percoll density gradient (63% and 73%) in buffer A. Mononuclear cells were subsequently separated from polymorphonuclear cells by centrifugation at $1200 \times g$ for 25 min at 18°C. Using this procedure, mononuclear cells were recovered from the 50%/63% Percoll interface.

Mononuclear cells were washed three times in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (HBSS) to remove Percoll and finally suspended in Ca^{2+}/Mg^{2+}-free HBSS at a concentration of 10^6 ml^{-1}. Cells (5×10^5) were added to 24-well culture plates (Greiner Labortecnik Ltd, Dursley, Gloucestershire) containing 500 μl Dutch-modified RPMI 1640 (RPMI 1640 supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) and allowed to adhere to the plastic for 90 min at 37°C in a humidified incubator under an atmosphere of 5% CO_2. The purity of the adherent cell population was routinely >94% monocytes. Plates were agitated, non-adherent cells decanted and the resulting monocytes were cultured for various times (see text and figure legends for details) in 1 ml supplemented Dutch-modified RMPI 1640 in the absence and presence of the drugs under investigation. The GM-CSF released into the culture supernatant was subsequently measured by an amplified sandwich ELISA.

Measurement of GM-CSF

Ninety-six well round-bottom plates were coated with 50 μl of a rat, anti-human GM-CSF monoclonal antibody diluted 1 : 250 in buffer B (in mM: $NaHCO_3$ 100, NaN_3 15, pH 8.2) and left overnight at 4°C. Plates were subsequently washed in buffer B and immediately blocked with 200 μl FCS (10% in buffer B) for 2 h at room temperature. After an additional wash with buffer B, 100 μl GM-CSF standards, quality controls and unknown samples, in supplemented Dutch-modified RPMI 1640, were added to the plates and left for 18 h at 4°C. Plates were washed in buffer B, incubated for 45 min at room temperature with 100 μl of a biotinylated rat, anti-human monoclonal GM-CSF antibody diluted 1 : 500 in buffer B supplemented with 10% FCS, washed again, and then incubated for an additional 30 min at room temperature with 100 μl of avidin-peroxidase diluted 1 : 400 in buffer B (supplemented with 10% FCS). Plates were washed again and developed with 100 μl ABTS (2,2′-azino-*bis*-(3-ethylbenzothiazoline-6-sulphonic acid) substrate solution (0.55 mM ABTS; 0.1 M citric acid, pH 4.35; 0.1% (30%) H_2O_2). GM-CSF was measured colorimetrically at 405 nm and quantified by interpolation from a standard curve constructed to known concentrations of human recombinant GM-CSF. The detection limit of this assay is 16 pg ml^{-1}.

A previous study has established that exposure of human monocytes to bacterial LPS evokes a time- and concentration-dependent elaboration of GM-CSF with a $t_{1/2}$ and EC_{50} of 14.3 h and 390 pg ml^{-1} respectively (Meja *et al*., 2000). Unless stated otherwise, LPS was used in all experiments at 3 ng ml^{-1} (EC_{90}) and GM-CSF was measured in the culture supernatant 18 h after the addition of LPS (see Meja *et al*., 2000 for further details).

Semi-quantitative RT-PCR

Total RNA was extracted from 4×10^6 adherent monocytes using the Qiagen RNeasy mini kit according to the manufacturer's instructions and up to 1 μg was treated with DNaseI to remove contaminating genomic DNA. Five hundred nanograms of RNA were reverse transcribed in a total volume of 20 μl in 50 mM Tris-HCl (pH 8.3; 25°C) containing 10 mM $MgCl_2$, 500 μM spermidine, 10 mM DTT, 9 u AMV reverse transcriptase, 40 u RNase inhibitor, 0.5 μg random hexamers (Pharmacia, Uppsala, Sweden) and 1 mM deoxynucleotides. A RT-generated cDNA encoding the GM-CSF gene was amplified by PCR using specific primers designed from the reported primary sequences (Table 1) deposited with the GenBank data base. To confirm the integrity of RNA and equal loading of sample, RT-PCR analysis of the GAPDH gene was routinely performed using primers synthesized from the sequence described in Maier *et al*. (1990). PCR amplification (25 and 28 cycles for GAPDH and GM-CSF respectively) was conducted in a reaction volume of 25 μl containing 0.5 u *Taq* polymerase at a denaturing temperature of 94°C for 30 s, specific annealing temperature (Table 1) and an extension temperature of 72°C for 30 s. The cycle number, which achieved exponential amplification where product was proportional to starting cDNA, was determined empirically by performing PCR on an 'average' cDNA sample by combining cDNA from all samples within one experiment. PCR products were subsequently size-fractionated on 2% agarose/TAE gels, stained with ethidium bromide and visualized under UV light. To confirm identity with the published cDNA-sequences, the GM-CSF and GAPDH amplification products were cloned in to pGEM5z®-vectors (Promega, Southampton, U.K.) and double-stranded sequencing was performed using the T7 Sequenase 2.0 system (Amersham, Buckinghamshire, U.K.).

After agarose gel electrophoresis, Southern blotting and hybridization were performed to confirm the identity of PCR products and to check for possible genomic contamination. To quantify product formation, aliquots (5 μl) of the PCR-product were 'dot-blotted' and hybridized with the appropriate radiolabelled cloned cDNA. After washing at high stringency the radioactivity associated with each 'dot-blot' was determined by Cérenkov counting. GM-CSF transcripts are expressed as ratio to GAPDH and relative values are plotted as means ± s.e.mean.

We have previously established that exposure of human monocytes to bacterial LPS (3 ng ml^{-1}) evokes a time-dependent accumulation of GM-CSF mRNA transcripts with a $t_{1/2}$ of 0.8 h (Meja *et al*., 2000). In all experiments LPS was used at 3 ng ml^{-1} and GM-CSF mRNA was measured 3 h after stimulation (see Meja *et al*., 2000 for further details).

Drugs and analytical reagents

Percoll was obtained from Pharmacia/LKB (Milton Keynes, Buckinghamshire, U.K.), and LPS (from *Salmonella enteritidis*), 8-Br-cyclic AMP, salbutamol, PGE_2, avidin-peroxidase (code A3151), FCS, ABTS and HBSS were from the Sigma Chemical Company (Poole, Dorset, U.K.). GM-CSF standards (code RH-CSF-C) and quality controls (code 88/646) were obtained from Genzyme and the National Institute for Biological Standards and Controls respectively. Rat, anti-human GM-CSF and biotinylated rat, anti-human GM-CSF monoclonal antibodies (codes 118551D and 18526D respectively) were purchased from Pharmingen/Cambridge Bioscience (Cambridge, U.K.). Fluorescein-conjugated streptavidin (code F0422) and biotinylated swine, anti-rabbit IgG (code E0353) were from Dako (Cambridge, U.K.). Human recombinant IL-10 (code 217-IL-005), IL-10 standards (code 217-IL-005) and PCR primers for GM-CSF and GAPDH genes were obtained from R & D systems Europe Ltd. (Abingdon, Oxfordshire, U.K.). Alkaline phosphatase-labelled sheep, anti-rabbit IgG antibodies were purchased from Stratech Scientific Ltd (Luton, Bedfordshire, U.K.). *Taq* DNA-polymerase was from Bioline (Finchley, London, U.K.), L-glutamine and RPMI 1640 were from Gibco (Rickmansworth, Hertfordshire), and AMV reverse transcriptase, RNasin®-ribonuclease-inhibitor and deoxynucleotides were purchased from Promega (Southampton, Hampshire, U.K.). Multiprime® DNA-labelling system and nitrocellulose membranes were supplied by Amersham International (Little Chalfont, Buckinghamshire, U.K.). Rolipram, Ro 20–1724, benafentrine, piclamilast, and SK&F 95654 and denbufylline were provided by Schering (Berlin, Germany), Calbiochem (Nottingham, U.K.), Byk-Gulden (Konstanz, Germany), Rhone-Poulenc Rorer (Dagenham, Essex, U.K.) and GlaxoSmithKline (King of Prussia, U.S.A.) respectively. All other reagents were from BDH (Poole, Dorset, U.K.).

Data and statistical analyses

Data points, and values in the text and figure legends, represent the mean ± s.e.mean of n independent determinations. Concentration-response curves were analysed by least-squares, non-linear iterative regression with the 'PRISM' curve fitting program (GraphPad, San Diego, California, U.S.A.) and EC_{50}/IC_{50} values were interpolated from curves of best-fit. When statistical evaluation was required, data were analysed by Student's t-test for paired data or by one-way ANOVA/Newman-Keuls multiple comparison test. The null hypothesis was rejected when $P < 0.05$.

Table 1 Primers and conditions used in RT-PCR experiments

Gene product	*GenBank Accession Number*	*Deoxyoligonucleotide sequences*	*Co-ordinates of PCR product in human cDNA sequence*	*Product size (base pairs)*	*Annealing temperature*
GM-CSF	P04141	Forward: 5′-CCA-TTC-TTC-TGC-CAT-GCC-TG-3′ Reverse: 5′-ATG-TTT-GAC-CTC-CAG-GAG-CCG-3′	116 to 615	500	58° for 30 s
GAPDH	J04038	Forward: 5′-TCT-AGA-CGG-CAG-GTC-AGG-TCC-ACC-3′ Reverse: 5′-CCA-CCC-ATG-GCA-AAT-TCC-ATG-GCA-3′	146 to 743	598	65° for 30 s

Results

Effect of rolipram and other isoenzyme-selective PDE inhibitors on LPS-induced GM-CSF release

Pre-treatment (20 min) of monocytes with the PDE4 inhibitor, rolipram (0.2, 2 and 20 μM) produced in a non-competitive inhibition of LPS (100 pg ml^{-1} to 1 μg ml^{-1})-induced GM-CSF generation with respect to vehicle-treated cells (Figure 1a). Thus, there was a progressive, concentration-related reduction in the maximum response without any change in the potency of LPS (EC_{50} values = 0.28, 0.31, 0.17 and 0.5 ng ml^{-1} for vehicle and 0.2, 2 and 20 μM rolipram respectively). At a fixed concentration of LPS (3 ng ml^{-1}), rolipram inhibited GM-CSF release in a concentration-dependent manner (EC_{50} ~1.8 μM) reducing output by ~80% at the maximally effective concentration (Figure 1b; Table 2).

Pre-treatment (20 min) of monocytes with the PDE inhibitors Ro 20–1724, denbufylline, piclamilast (all PDE4-selective) and benafentrine (PDE3/4-selective) inhibited LPS-induced GM-CSF release in a concentration-dependent manner with EC_{50} values of 41, 3.5, 0.009 and 22 μM respectively (Figure 2; Table 2). A selective inhibitor of PDE3 (SK&F 95654) did not affect LPS-induced GM-CSF generation at concentrations where isoenzyme-selectivity is preserved (data not shown).

Effect of 8-Br-cyclic AMP and cyclic AMP-elevating drugs on LPS-induced GM-CSF release

Exposure (30 min) of human monocytes to 8-Br-cyclic AMP suppressed LPS-induced GM-CSF release in a concentration-dependent manner with an IC_{50} of 271 μM (Figure 3a; Table 2). Maximum inhibition was achieved at 10 mM 8-Br-cyclic AMP, a concentration that inhibited cytokine generation by >90%. Similarly, incubation (2 h) of monocytes with cholera toxin (CTX) prior to the addition of LPS reduced the release of GM-CSF by 54% at the maximally effective concentration (Figure 3b).

Figures 3c,d show the effect of salbutamol and PGE_2 on LPS-induced GM-CSF generation. Contrary to its inhibitory effect on TNFα secretion (Figure 3c – dotted line; Seldon *et al*., 1995) salbutamol (5 min pre-treatment) did not affect the elaboration of GM-CSF at any concentration examined. In contrast PGE_2, which acts through an inhibitory EP_2-like receptor on monocytes (Meja *et al*., 1997), reduced GM-CSF output in a concentration-dependent manner with an IC_{50} of 307 nM (Figure 3d).

Reduced sensitivity of LPS-induced GM-CSF release to cyclic AMP

A consistent finding of these studies was that the LPS-driven release of GM-CSF was significantly less sensitive (four- to 13-times) to all of the PDE inhibitors studied when compared to the elaboration of TNFα (dotted lines) under identical experimental conditions (Figures 1b, 2) (Seldon *et al*., 1995). Moreover, GM-CSF release was never abolished (*cf*. TNFα). Indeed, a maximally effective concentration of piclamilast (100 nM), denbufylline (100 μM) and benafentrine (100 μM) suppressed GM-CSF by only ~50, 70 and 80% respectively (Figure 2d). CTX, PGE_2 and 8-Br-cyclic AMP were also less potent and less effective at inhibiting the release of GM-CSF than of TNFα, while salbutamol was inactive (Figure 3, dotted line; see Table 2).

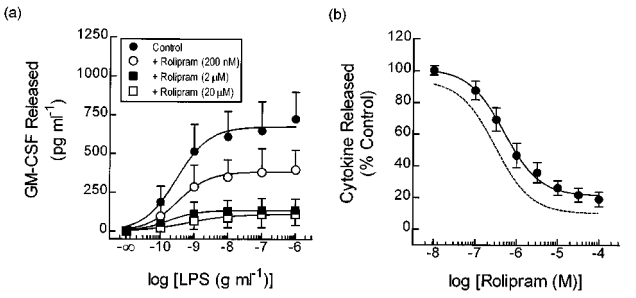

Figure 1 Effect of rolipram on LPS-induced GM-CSF release. Adherent monocytes were treated for 20 min with rolipram and then exposed to LPS. Cells were maintained at 37°C in a thermostatically-controlled incubator under a 5% CO_2 atmosphere and the amount of GM-CSF released into the culture supernatant was measured at 18 h by ELISA. The effect of rolipram on the LPS concentration-response relationship and at a fixed submaximal concentration of LPS (3 ng ml^{-1}) is shown in (a) and (b) respectively. The dotted line in (b) shows the position of the concentration-response curve that describes the inhibition of TNFα generation under identical experimental conditions (data taken from Seldon *et al*., 1995; 1998b). Each data point represents the mean ± s.e.mean of nine independent determinations.

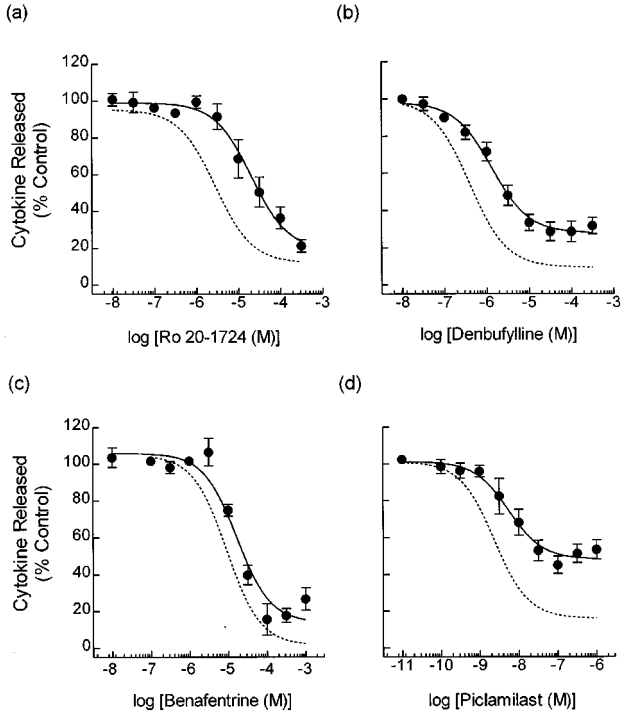

Figure 2 Effect of PDE4 inhibitors on LPS-induced GM-CSF release. Monocytes were treated with Ro 20-1724 (a), denbufylline (b), benafentrine (c) and piclamilast (d), exposed to LPS and the amount of GM-CSF released into the culture supernatant was measured by ELISA. The dotted line in each panel shows the position of the concentration-response curve that describes the inhibition of TNFα generation (data taken from Seldon *et al*., 1995; 1998b). Each data point represents the mean ± s.e.mean of three to six independent determinations.

Table 2 Effect of cyclic AMP PDE inhibitors and other drugs on LPS-induced GM-CSF and TNFα generation from human peripheral blood monocytes

Drug	*GM-CSF* (IC_{50}-μM)	*Maximum inhibition* (%)	*TNFα** (IC_{50}-μM)	*Maximum inhibition* (%)	*Selectivity GM-CSF IC_{50}/TNFα IC_{50}*
Rolipram	1.79±0.7 (9)	81	0.41	93	4.4
Ro 20-1724	40.9±22.0 (4)	79	3.2	95	12.9
Denbufylline	3.48±1.11 (5)	71	0.34	98	10.2
Benafentrine	22.0±3.00 (3)	82	5.5	97	4
Piclamilast**	0.0088±0.0031 (6)	46	0.0024	82	3.7
SK&F 95654	>300 (3)	–	>100	–	–
Salbutamol	Inactive	–	0.049	41	–
PGE_2	0.31±0.04 (4)	77	0.077	93	3.9
8-Br-cyclic AMP	271.4±49.5 (5)	89	101	100	2.7
CTX**	338.5±24.0 (3)***	54	143	95	2.4

Adherent monocytes were pre-treated with the drugs indicated above and then exposed to LPS (3 ng mg^{-1}). Cells were maintained at 37°C in a thermostatically-controlled incubator under a 5% CO_2 atmosphere and the amount of GM-CSF and TNFα released into the culture supernatant was measured at 18 h by immunospecific ELISAs. IC_{50} values represent the mean±s.e.mean of *n* determinations shown in parentheses. See Methods for further details. *Data taken from Seldon *et al*., 1995; 1998b. **EC_{50} given as maximum inhibition of GM-CSF release less than 50%. ***Value in pg ml^{-1}.

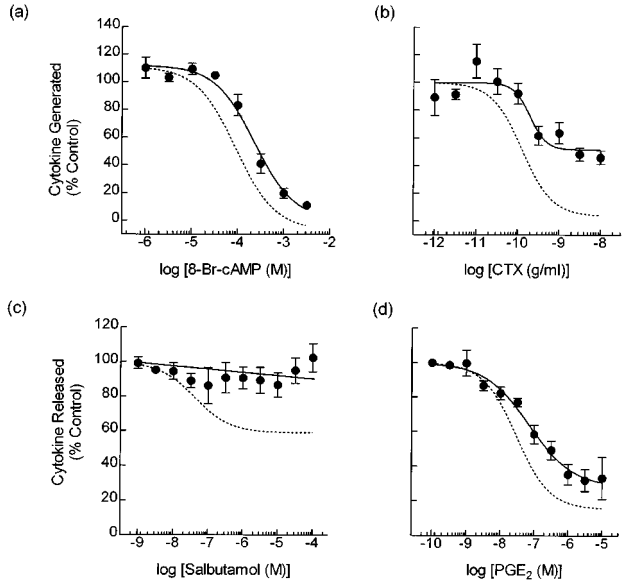

Figure 3 Effect of other cAMP-elevating drugs on LPS-induced GM-CSF release. Monocytes were treated with 8-Br-cyclic AMP (30 min; panel a), CTX (2 h; b), salbutamol (5 min; c), or PGE_2 (5 min; d) exposed to LPS and the amount of GM-CSF released into the culture supernatant was measured by ELISA. The dotted line in each panel shows the position of the concentration-response curve that describes the inhibition of TNFα generation (data taken from Seldon *et al*., 1995; 1998b). Each data point represents the mean±s.e.mean of three to five independent determinations.

Effect of rolipram, 8-Br-cyclic AMP, salbutamol and PGE_2 on GM-CSF mRNA expression

In unstimulated adherent monocytes GM-CSF mRNA transcripts were present in a very low copy number as assessed by RT-PCR (28 cycles of amplification). However, 3 h after stimulation of monocytes with LPS (3 ng ml^{-1}) GM-CSF mRNA expression was increased 51 fold relative to the 'house-keeping' gene GAPDH (Figure 4). Pre-treatment of monocytes with PGE_2 (1 μM; 5 min), 8-Br-cyclic AMP (1 mM; 30 min) and rolipram (10 μM; 20 min) significantly attenuated this response by 44, 58 and 57% respectively (Figure 4), whereas salbutamol (1 μM; 5 min) was without effect on GM-CSF mRNA expression (Figure 4).

Effect of IL-10 on LPS-induced GM-CSF generation and reversal by an anti-IL-10 antibody

Pre-treatment of monocytes with human recombinant (hr) IL-10 suppressed LPS-induced GM-CSF release in a concentration-dependent manner (IC_{50}=71.2±19.1 pg ml^{-1}) with complete inhibition achieved between 3–10 ng ml^{-1} (Figure 5). The introduction of an anti-IL-10 neutralizing antibody to monocyte cultures attenuated the inhibitory effect of hrIL-10 (1 ng ml^{-1}) in a concentration-dependent manner (EC_{50}=9.5±0.9 ng ml^{-1}) with complete reversal at 0.1–1 μg ml^{-1} (Figure 5). An isotype-matched control antibody (rat IgG_{1k}) failed to block the inhibitory effect of hrIL-10 (data not shown).

Effect of an anti-IL-10 antibody on LPS-induced GM-CSF generation

Pre-treatment (20 min) of monocytes with an anti-IL-10 antibody at 3, 30 and 300 ng ml^{-1} had no effect on the potency of LPS for GM-CSF generation (EC_{50}'s=0.24±0.07, 0.27±0.07 and 0.33±0.1 ng ml^{-1} respectively) when compared to untreated cells (EC_{50}=0.23±0.07; Figure 6). However, the anti-IL-10 antibody, but not rat IgG_{1k}, significantly augmented the maximum amount of GM-CSF released confirming previous reports that IL-10 is generated in response to LPS (Seldon *et al*., 1998b) and acts in a negative autocrine factor to temper the production of GM-CSF. This effect was concentration-related such that LPS (100 ng ml^{-1})-induced GM-CSF release was augmented 1.4 fold from 547±89 to 769±117 pg ml^{-1} ($P<0.05$) in the presence of 300 ng ml^{-1} of the anti-IL-10 antibody (Figure 6). The potentiation of GM-CSF release by the anti-IL-10 antibody varied markedly (14–195%) between subjects (mean=69±22%, $n=18$), which presumably reflects the significant variation in monocyte IL-10 production between donors (Platzer *et al*., 1995; Seldon *et al*., 1998b).

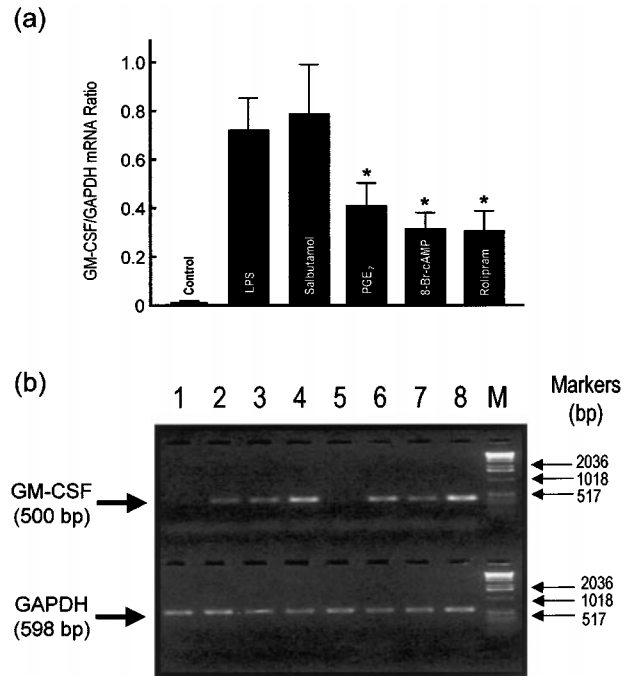

Figure 4 Effect of cyclic AMP-elevating drugs on LPS-induced GM-CSF mRNA expression. Adherent monocytes were pre-treated with salbutamol (5 min; 1 μM), PGE_2 (5 min; 1 μM), 8-Br-cyclic AMP (30 min; 1 mM), rolipram (20 min; 10 μM) or vehicle and exposed to LPS (3 ng ml^{-1}). After 3 h, RNA was extracted and 0.5 μg was reversed transcribed to generate cDNAs for GM-CSF and GAPDH using the primer pairs shown in Table 1. PCR was performed with reverse transcribed cDNA, the products subjected to electrophoresis on 1.5% agarose gels and DNA subsequently visualized after staining with ethidium bromide. PCR products were quantified by Southern blotting and standardized against GAPDH. RT-PCR product sizes for GM-CSF and GAPDH were 500 bp (28 cycles) and 598 bp (24 cycles) respectively. (a) and (b) show the mean data of four independent experiments and a representative gel (prior to Southern hybridization) respectively. 1, Control; 2, LPS + Rolipram; 3, LPS + 8-Br-cyclic AMP; 4, LPS; 5, Control; 6, LPS + Salbutamol; 7, LPS + PGE_2; 8, LPS. *$P < 0.05$, significant inhibition of LPS-induced GM-CSF mRNA expression.

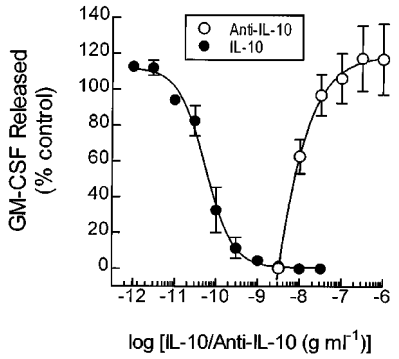

Figure 5 Inhibition of LPS-induced GM-CSF release by hrIL-10 and reversal by anti-IL-10. Adherent monocytes were pre-treated (20 min) with hrIL-10, or hrIL-10 in the presence of anti-IL-10 before being exposed to LPS (3 ng ml^{-1}). The amount of GM-CSF released in to the culture supernatant was then measured by ELISA at 18 h. Each data point represents the mean ± s.e.mean of four independent determinations.

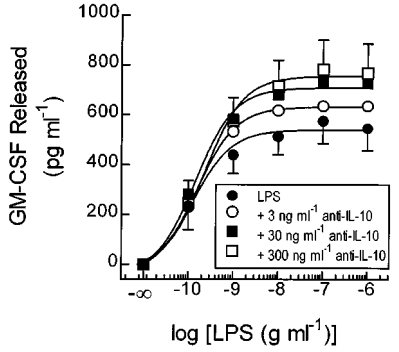

Figure 6 Effect of an anti-IL-10 antibody on LPS-induced GM-CSF release. Adherent monocytes were pre-treated (20 min) with anti-IL-10 (as indicated) before being exposed to LPS. The amount of GM-CSF released in to the culture supernatant was then measured by ELISA at 18 h. Each data point represents the mean ± s.e.mean of four independent determinations.

Inhibition of LPS-induced GM-CSF generation by rolipram, PGE_2 and 8-Br-cyclic AMP: effect of an anti-IL-10 antibody

Pre-treatment of monocytes with rolipram, PGE_2 and 8-Br-cyclic AMP inhibited LPS-induced GM-CSF release in a concentration-dependent manner (Figure 7). In the presence of a concentration (100 ng ml^{-1}) of the anti-IL-10 antibody that abolished the inhibitory action of a maximally effective concentration of exogenous hrIL-10, there was an upwards shift of the concentration-response curves that described the inhibition of GM-CSF by each drug without a significant change in their potency. As a consequence, the maximum percentage inhibition of GM-CSF release was significantly attenuated (Figure 7; Table 3). Since the anti-IL-10 antibody significantly augmented LPS-induced GM-CSF release it was reasoned that the reduced ability of cyclic AMP to antagonize inhibitory effect of LPS might be due to functional antagonism. To address this possibility, experiments were conducted at different concentrations of LPS in the absence and presence of anti-IL-10 (100 ng ml^{-1}). As shown in Table 4, the maximum inhibition of GM-CSF release effected by rolipram, PGE_2 and 8-Br-cyclic AMP was identical at all concentrations of LPS studied.

Discussion

In the present study we have confirmed previous investigations that human peripheral blood monocytes release the haematopoietic pro-inflammatory cytokine, GM-CSF in response to bacterial LPS (Blanchard *et al*., 1991; de Waal Malefyt *et al*., 1991; Meja *et al*., 2000). In addition, we have extended this finding by providing evidence that cyclic AMP-elevating drugs act at both the transcriptional and post-transcriptional level to suppress GM-CSF output by a mechanism that is not mediated by IL-10.

Effect of cyclic AMP-elevating drugs on LPS-induced GM-CSF mRNA expression and protein release

Pre-treatment of monocytes with the PDE inhibitors rolipram, Ro 20-1724, denbufylline, piclamilast and benafentrine inhibited LPS-induced GM-CSF release with a rank

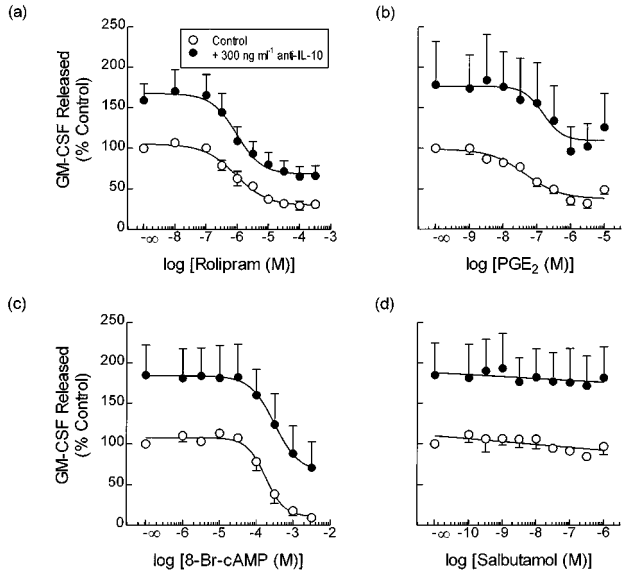

Figure 7 Effect of an anti-IL-10 antibody on the inhibitory effect of cyclic AMP-elevating drugs on LPS-induced GM-CSF release. Monocytes were treated with rolipram (a), PGE_2 (b), 8-Br-cyclic AMP (c) and salbutamol (d) in the absence and presence of anti-10 (100 ng ml^{-1}), exposed to LPS (3 ng ml^{-1}) and the amount of GM-CSF released into the culture supernatant was measured by ELISA. Each data point represents the mean$\pm$s.e.mean of three to six independent determinations.

order of potency similar to that reportedly previously for the inhibition of PDE4 in monocyte lysates (Seldon *et al*., 1995). These results are consistent with PDE4 being the major cyclic AMP hydrolyzing activity in monocytes (Gantner *et al*., 1997; Seldon *et al*., 1995) and the ability of PDE4 inhibitors to attenuate GM-CSF release from human bone marrow stromal cells and T-lymphocytes (Borger *et al*., 1996; Bug *et al*., 1998). In contrast, a selective inhibitor of PDE3 (SK&F 95654) did not affect LPS-induced GM-CSF generation at concentrations where isoenzyme-selectivity is preserved. Although appreciable PDE3 is present in the particulate fraction of human monocytes (Gantner *et al*., 1997), these data show that inhibition of this enzyme family does not regulate the release of GM-CSF, which is in agreement with the inability of PDE3 inhibitors to suppress the elaboration of TNFα and IL-2 from monocytes (Seldon *et al*., 1995) and mitogen-stimulated T-lymphocytes respectively (Giembycz *et al*., 1996).

Further studies were performed to assess whether agonists (PGE_2 and salbutamol) that can act *via* Gs-coupled receptors could also suppress GM-CSF generation. Unexpectedly, salbutamol did not affect the elaboration of GM-CSF at any concentration examined under conditions where PGE_2, which acts through an inhibitory 'EP_2-like' receptor on monocytes (Meja *et al*., 1997), reduced GM-CSF output. The reason for this discrepancy is unclear given that other cyclic AMP-elevating drugs were active in this system. As LPS-induced TNFα release is suppressed by salbutamol an absence of functional β_2-adrenoceptors cannot account for this difference (Seldon *et al*., 1995; 1998a). One possibility is that the EP_2-like receptor is coupled more efficiently to Gs/adenylyl cyclase on human monocytes than is the β_2-adrenoceptor. Equally, the density of EP_2-like receptors might be significantly greater than the number of β_2-adrenoceptors such that the cyclic AMP signal generated by salbutamol is relatively modest when compared to that effected by PGE_2. Both these ideas are supported by the finding that PGE_2 activates cyclic AMP-dependent protein kinase (PKA) to a significantly greater extent in human monocytes than salbutamol under identical experimental conditions (Seldon *et al*., 1995). Similarly, PGE_2-induced cyclic AMP accumulation and PKA activation are markedly potentiated by the PDE4 inhibitor, rolipram, when compared to the same responses elicited by salbutamol (Seldon *et al*., 1995). Taken together, these data suggest that the extent to which cyclic AMP is elevated and PKA activated might be primary determinants for inhibition of LPS-induced GM-CSF release from human monocytes.

A consistent finding of these studies was that the LPS-driven release of GM-CSF was significantly less sensitive (four- to 13-times) to all of the PDE inhibitors studied and the inhibition produced less when compared to the elaboration of TNFα (Seldon *et al*., 1995). Indeed, at maximally effective concentrations piclamilast, denbufylline and benafentrine suppressed the elaboration of GM-CSF by only $\sim$50, 70 and 80% respectively. This reduction in potency and activity held for other cyclic AMP-elevating drugs including PGE_2, CTX, and 8-Br-cyclic AMP suggesting that LPS regulates the expression of GM-CSF and TNFα by distinct mechanisms that display different sensitivities to cyclic AMP.

We have previously reported that LPS evokes a time-dependent and transient accumulation of GM-CSF mRNA transcripts in human monocytes that is abolished by actinomycin D and cycloheximide indicative of *de novo* transcription and translation of the GM-CSF gene (Meja *et al*., 2000). In unstimulated adherent monocytes GM-CSF mRNA is barely detectable but increases more than 50 fold after 3 h exposure of cells to LPS. In the present manuscript we have shown that pre-treatment of monocytes with PGE_2, 8-Br-cyclic AMP and rolipram prior to LPS significantly attenuated GM-CSF mRNA expression at 3 h by 40 to 60%. Other investigators have established that cyclic AMP-elevating drugs attenuate GM-CSF release in T-lymphocytes by reducing both the transcription rate of the GM-CSF gene and GM-CSF mRNA stability (Borger *et al*., 1996). Although either of these mechanism could equally account for the reduction in GM-CSF output from human monocytes, the finding that significant GM-CSF mRNA was present in 8-Br-cyclic AMP-, PGE_2- and rolipram-treated cells under conditions where GM-CSF release was suppressed by 80 to 90% indicates that cyclic AMP also impairs translation of GM-CSF mRNA to protein.

Pre-treatment of monocytes with salbutamol had no effect on GM-CSF mRNA expression despite clear activation of the cyclic AMP/PKA cascade in these cells (Seldon *et al*., 1995). This result is consistent with the lack of effect of salbutamol on GM-CSF output and endorses the contention that the magnitude of the cyclic AMP/PKA response may be critical in determining whether cytokine expression is repressed.

Role of IL-10 in GM-CSF expression

As described in the introduction there is evidence from both *in vitro* and *in vivo* studies that the generation of IL-10 by LPS-sensitive cells is enhanced by cyclic AMP, which acts in

Table 3 Effect of an anti-IL-10 antibody on the inhibition of LPS-induced GM-CSF generation by rolipram, 8-Br-cyclic AMP and PGE_2

		− Anti IL-10		*+ Anti-IL-10*	
	n	EC_{50} (μM)	*Maximum inhibition* (%)	EC_{50} (μM)	*Maximum inhibition* (%)
Rolipram	4	1.16±0.44	69.1±4.4	0.99±0.23	58.7±5.6*
8-Br-cyclic AMP	3	203.3±65.6	90.8±3.8	255.7±44.1	66.5±3.4*
PGE_2	3	0.062±0.10	51.1±6.8	0.16±0.08	30.9±4.1*

Adherent monocytes were pre-treated with the drugs indicated below in the absence and presence of anti-IL-10 (100 ng ml^{-1}) and then exposed to LPS (3 ng ml^{-1}). Cells were maintained at 37°C in a thermostatically-controlled incubator under a 5% CO_2 atmosphere and the amount of GM-CSF released into the culture supernatant was measured at 18 h by immunospecific ELISA. Data represent the mean±s.e.mean of *n* independent determinations. See Methods for further details. *$P<0.05$, significant reduction in the maximum inihbition of GM-CSF release in anti-IL-10-treated cells when compared to control.

Table 4 Effect of an anti-IL-10 antibody on the inhibitory effect of rolipram, 8-Br-cyclic AMP and PGE_2 on GM-CSF release at different concentrations of LPS

	Inhibition of LPS-induced GM-CSF release (%)					
	8-Br-cyclic AMP (1 mM)		*PGE_2* (1 μM)		*Rolipram* (30 μM)	
[LPS] (ng ml^{-1})	*− Anti-IL-10*	*+ Anti-IL-10*	*− Anti-IL-10*	*+ Anti-IL-10*	*− Anti-IL-10*	*+ Anti-IL-10*
0.3	74.1±14.7	57.6±14.3*	50.8±4.8	23.7±2.4*	71.3±10.2	55.4±9.9*
1	79.8±10.7	68.1±5.4	59.7±7.2	41.0±4.8*	74.9±5.9	64.2±8.2*
3	83.6±8.8	70.7±5.3	61.2±8.2	42.4±8.6*	75.7±3.7	59.4±5.8*
10	82.9±8.7	71.1±5.1*	63.3±8.7	40.9±11.3*	79.4±1.9	62.6±6.4*

Adherent monocytes were pre-treated with the drugs indicated below in the absence and presence of anti-IL-10 (100 ng ml^{-1}) and then exposed to LPS (0.3, 1, 3 or 10 ng ml^{-1}). Cells were maintained at 37°C in a thermostatically-controlled incubator under a 5% CO_2 atmosphere and the amount of GM-CSF released into the culture supernatant was measured at 18 h by immunospecific ELISA. Data represent the mean±s.e.mean of four independent determinations. See Methods for further details. *$P<0.05$. Significantly less inhibition of GM-CSF generation in the presence of antil-IL-10. Student's paired *t*-test.

an autocrine manner to inhibit pro-inflammatory cytokine production (Arai *et al*., 1995; Jilg *et al*., 1996; Kambayashi *et al*., 1995; Strassmann *et al*., 1994). To establish whether such a mechanism could account for the inhibitory effect of cyclic AMP on GM-CSF production in human monocytes the effect of neutralising IL-10 was studied. As shown in Figure 7 an anti-IL-10 antibody, at a concentration that abolished the inhibitory action of a maximally effective concentration of exogenous hrIL-10, produced a parallel upwards shift of the concentration-response curves that described the inhibition of GM-CSF release by rolipram, PGE_2 and 8-Br-cyclic AMP without changing their potency. Although the maximum percentage inhibition of GM-CSF release was reduced this was not due to inhibition of the effect of rolipram, PGE_2 and 8-Br-cyclic AMP but merely a reflection of the higher starting baseline. Curiously, these results are contrary to comparable studies performed with murine macrophages (Kambayashi *et al*., 1995; Strassmann *et al*., 1994), Kuppfer cells (Arai *et al*., 1995) and *in vivo* murine models of endotoxin shock (Arai *et al*., 1995; Jilg *et al*., 1996). In each of these experimental settings an anti-IL-10 antibody was reported to abolish or markedly attenuate the protection of LPS-evoked TNFα and IL-6 production, liver injury and lethality afforded by agents that elevate cyclic AMP.

Currently, it is unclear why anti-IL-10 reverses the inhibitory effect of cyclic AMP-elevating drugs on cytokine production from LPS-sensitive cells of murine origin but not from human monocytes. We have previously reported that this disparity is not due to a failure of monocytes to release IL-10 or the ability of cyclic AMP-elevating agents to augment this response (Seldon *et al*., 1998). Dissimilar kinetics of IL-10 *versus* GM-CSF release also cannot explain this difference (Meja *et al*., 2000; Seldon *et al*., 1998b). However, the possibility that transcriptional/post-transcriptional control of certain pro-inflammatory cytokine genes varies between humans and mice, and/or monocytes and macrophages is worthy of consideration and is currently being assessed in the authors' laboratory.

The upwards shift of the concentration-response curves that described the suppression of LPS-induced GM-CSF generation by 8-Br-cyclic AMP, PGE_2 and rolipram in anti-IL-10-treated monocytes was parallel such that the maximum response produce by these drugs was significantly reduced (Table 3). It was reasoned that one explanation for the reduced effect was functional antagonism and this possibility was addressed by performing experiments at different concentrations of LPS in the absence and presence of a neutralizing anti-IL-10 antibody. However, as shown in Table 4 the maximum inhibition of GM-CSF release effected by rolipram, PGE_2 and 8-Br-cyclic AMP was identical at all concentrations of LPS studied indicating that in anti-IL-10-treated monocytes only about 50% of the GM-CSF released by LPS is sensitive to inhibition by cyclic AMP.

In conclusion, the results of the present study demonstrate unequivocally that cyclic AMP-elevating drugs attenuate the elaboration of GM-CSF from LPS-stimulated human monocytes by a mechanism that is not mediated *via* the inhibitory cytokine, IL-10. As GM-CSF primes eosinophils and neutrophils and enhances their survival, suppression of this cytokine from monocytes/macrophages in the airways could explain, at least in part, the reported efficacy of PDE4 inhibitors clinical trials of COPD (Giembycz, 2001; Torphy *et al*., 1999).

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