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1 **Ciclesonide Inhibits TNFα and IL-1β Induced Monocyte Chemotactic Protein-1 (MCP-**
2 **1/ CCL2) Secretion from Human Airway Smooth Muscle Cells**

3

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15 Ciclesonide inhibition of TNFα and IL-1β induced MCP-1

16

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18 J.K. Patel designed and performed experiments, R.L. Clifford wrote the paper, designed and
19 performed experiments, K Deacon designed and performed experiments, A.J. Knox initiated
20 and directed the study.

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21 **Abstract**

22 MCP-1 is a member of the CC family of cytokines. It has monocyte and lymphocyte
23 chemotactic activity and stimulates histamine release from basophils. MCP-1 is
24 implicated in the pathogenesis of inflammatory diseases including asthma. The airway
25 smooth muscle layer is thickened in asthma and the growth factors and cytokines airway
26 smooth muscle (ASM) cells secrete play a role in the inflammatory response of the
27 bronchial wall. Glucocorticoids and β_2-agonists are frontline drug treatments for asthma.
28 Little is known about the effect of asthma treatments on MCP-1 production from human
29 ASM cells. Here we determined the effect of Ciclesonide (glucocorticoid) and
30 Formoterol (β_2 agonist) on MCP-1 production from human ASM cells. TNFα and IL-1β
31 induced MCP-1 secretion from human ASM cells. Formoterol had no effect on MCP-1
32 expression while Ciclesonide significantly inhibited both IL-1β and TNFα induced MCP-
33 1. Furthermore, Ciclesonide inhibited IL1β and TNFα induced MCP-1 mRNA and IL-1β
34 and TNFα induced MCP-1 promoter and enhancer luciferase reporters. Western blots
35 showed that Ciclesonide had no effect on IκB degradation. Finally Ciclesonide inhibited
36 an NF-κB luciferase reporter. Our data shows that Ciclesonide inhibits IL-1β and TNFα
37 induced MCP-1 production from human ASM cells via a transcriptional mechanism
38 involving inhibition of NFκB binding.

39

40 Glucocorticoid, NFκB, Inflammation

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42

43

44 **Introduction**

45 MCP-1 (CCL2) is a member of the CC family of cytokines. It is a three-exon gene,
46 regulated by a distal enhancer and proximal promoter region, separated by 2.2kb of DNA
47 (18). The proximal promoter region is just upstream of the transcription start site and
48 contains a GC box that binds Sp1, an NFκB and an NF-1 binding site in addition to two
49 AP-1 sites. The distal enhancer contains two nuclear factor-κB (NF-κB) binding sites
50 (3).

51

52 MCP-1 has monocyte and lymphocyte chemotactic activity and can stimulate histamine
53 release from basophils (13). It is implicated in a number of inflammatory diseases
54 including asthma. Asthma is a chronic inflammatory disease characterised by increased
55 lamina reticularis, smooth muscle cell hyperplasia, airway hyperreactivity and airway
56 inflammation (15). Staining of asthmatic and non-asthmatic bronchial biopsies shows
57 the presence of MCP-1 in the bronchial epithelium, subepithelial macrophages, blood
58 vessels and bronchial smooth muscle, with stronger MCP-1 staining in the asthmatic
59 epithelium and subepithelial layer (24). MCP-1 is also present in bronchoalveolar lavage
60 fluid (BALF) from healthy and atopic asthma patients, with higher concentrations in the
61 asthmatic BALF. Furthermore asthmatic levels of BALF MCP-1 inversely correlate with
62 measures of pulmonary function, namely FEV1 and FEF_{50} (13). Allergen challenge can
63 further increase BALF MCP-1 levels (12). Serum MCP-1 levels are also increased in
64 asthmatic samples compared to non-asthmatic, and asthmatic levels increase further
65 during an acute asthma attack (4). Expression of MCP-1 in murine lungs coincides with
66 leukcocyte infiltration in an OVA model of induced lung allergic inflammation.

67 Blockade of MCP-1 in the OVA model reduces migration of macrophages, monocytes, T
68 lymphocytes and eosinophils to the lung in response to OVA and the induction of
69 bronchial hyperreactivity (10). MCP-1 blockade also reduces OVA induced levels of
70 leukotriene B4 (LTB_4), prostaglandin E2 (PGE_2), thromboxane B2 (TXB_2), interleukin-5
71 (IL-5) and interleukin-4 (IL4), suggesting that MCP-1 is involved in B and T cell
72 activation (10). Interestingly a polymorphism in the enhancer region of MCP-1 gene is
73 associated with asthma in children (26).

74

75 The grossly thickened airway smooth muscle is an important cellular source of
76 chemokines and growth factors in asthma (9). Smooth muscle cells are considered to
77 play a central role in orchestration of the inflammatory response within the bronchial wall
78 (6). MCP-1 secretion from human ASM cells was first described in 1998 (28) and since
79 then its regulation by various stimuli has been investigated. Interleukin 1β (IL-1β), tumor
80 necrosis factor α (TNFα) and endothelin-1 (ET-1) stimulate MCP-1 mRNA production
81 and protein secretion from human ASM cells (16, 25, 28). Pharmacological inhibitor
82 studies suggest IL-1β induced MCP-1 expression is dependent on p38 MAPK, JNK
83 kinase, p42/44 ERK and the transcription factor NF-κB (29). Detailed characterisation of
84 ET-1 induced MCP-1 expression shows a requirement for p38 and p42/44 MAPK
85 signalling and a transcriptional mechanism involving binding of AP-1 c-Jun subunit and
86 NF-κB p65 subunit to the MCP-1 promoter, with little effect on the MCP-1 enhancer
87 (25). The mechanism of TNFα induced MCP-1 expression in human ASM cells has not
88 been described. However in NIH 3T3 fibroblasts TNFα induced MCP-1 requires a
89 sequential series of events that links the proximal promoter and distal enhancer regions.

90 TNFα induction of NF-κB binding to the distal enhancer recruits the histone acetyl
91 transferase (HAT) p300/CBP. P300/CBP acetylates both the distal enhancer and
92 proximal promoter associated histones. Proximal histone acetylation allows access for
93 Sp1 binding to the proximal promoter and subsequent transcription (27).
94

95 Glucocorticoids and β_2-agonists are commonly used for asthma therapy due to their anti-
96 inflammatory and bronchodilatory effects respectively. The glucocorticoid
97 Dexamethasone inhibits cytomix (combination if IL-1β, TNFα and interferon γ (IFNγ))
98 induced MCP-1 mRNA and protein secretion (21) while Fluticasone inhibits TNFα
99 induced MCP-1 via an undefined posttranscriptional mechanism (16). The β_2 agonist
100 Salmeterol has no effect on TNFα induced MCP-1 expression (16). No further
101 information regarding MCP-1 regulation by glucocorticoids or β_2 agonists exists. The
102 purpose of this study was to investigate the effect of the glucocorticoid Ciclesonide and
103 β_2 agonist Formoterol on IL-1β and TNFα induced MCP-1 expression from human ASM
104 cells. We found that Ciclesonide inhibited both TNFα and IL-1β induced MCP-1
105 expression while Formoterol had no effect. The Ciclesonide effect was mediated via the
106 MCP-1 promoter and enhancer regions and regulation of NF-κB binding.
107

108 **Materials and Methods**

109 **Materials**

110 Formoterol fumarate dehydrate, 5-(p-Fluorophenyl)-2-ureido]thiophene-3-carboxamide
111 (TPCA-1), Dulbecco's modified Eagle's medium (DMEM), L-glutamine, Amphotericin
112 B, Penicillin, Streptomycin and Trypsin-EDTA were purchased from Sigma, Poole,

113 Dorset, UK. Ciclesonide was a gift from Altana Pharma AG, Konstanz, Germany.
114 Recombinant Human Interleukin 1beta (rhIL-1β), Recombinant Human Tumour Necrosis
115 Factor Alpha (rhTNFα) and DuoSet®ELISA development System human CCL2/MCP-1
116 were purchased from R&D Systems, Abingdon, Oxfordshire, UK. All reagents for firefly
117 luciferase assay system were purchased from Promega, Southampton, Hampshire, UK.
118 FuGene 6 transfection reagent was from Roche Molecular Biochemicals, Lewes, East
119 Sussex, UK. Rainbow™ coloured protein molecular weight marker, ECL western
120 blotting detection reagent and Hyperfilm™-ECL were from Amersham,
121 Buckinghamshire, UK. Nitrocellulose membrane was purchased from Bio-Rad, Hemel
122 Hemstead, Hertfordshire, UK. Polyclonal goat anti-rabbit antibody conjugated to
123 horseradish peroxidase was from DakoCytomation, Ely, Cambridge, UK. Phospho IκB-α
124 (Ser 32) and IκB-α were purchased from Cell signalling/New England Biolabs, Hitchin,
125 Hertfordshire, UK. The NucleoSpin RNA II kit was purchased from Fisher Scientific,
126 Loughborough, Leicestershire, UK. Moloney murine leukemia virus reverse
127 transcriptase, RNase inhibitor, $(dT)_{15}$ primer, dNTPs and Moloney murine leukemia virus
128 RT buffer were purchased from Promega. TaKaRa SYBR *Premix Ex Taq* was purchased
129 from Lonza Group Ltd., Basel, Switzerland. MB120L was a kind gift from
130 GlaxoSmithKline

131

132 **Cell culture**

133 Human ASM cells were from human tracheas obtained from post-mortems as previously
134 described (Pang & Knox, 1997). Primary normal human ASM cells were grown in
135 DMEM supplemented with 4mM L-glutamine, 2.5μg/ml Amphotericin B, 100 U/ml

136 penicillin, 100μg/ml Streptomycin and 10% heat inactivated foetal bovine serum in
137 humidified 5% CO_2/95% air at 37°C. Cells at passage 6-7 were used for all experiments.
138

139 **Experimental Protocols**

140 Once fully confluent the cells were growth arrested in DMEM serum free media and
141 incubated at 37°C for 24 hours prior to treatment. Cells were then treated with IL-1β (0-
142 10ng/ml^{-1}) or TNFα (0-100ng/ml^{-1}) for 24 hours in concentration response experiments.
143 The supernatants were then assayed for MCP-1. In inhibitor studies cells were pre-
144 incubated for 30mins prior to IL-1β or TNFα treatment for 24 hours. Vehicle (DMSO)
145 was added to control wells at equivalent concentration (0.1% maximum concentration).
146

147 **MCP-1 ELISA**

148 MCP-1 DuoSet ELISA kit was used to measure MCP-1 concentrations in cell culture
149 supernatants according to the manufacturer's protocol. All cell supernatants were diluted
150 either 1:50 or 1:200 in reagent diluent to enable all concentrations to be within the
151 standard curve.
152

153 **RNA Isolation and Reverse Transcription**

154 Total RNA was isolated using the NucleoSpin RNA II kit following the manufacturers
155 protocol. 5μl of RNA was reverse-transcribed in a total volume of 25μl, including 132
156 units of Moloney murine leukemia virus reverse transcriptase, 26.4 units of RNase
157 inhibitor, 0.6μg of $(dT)_{15}$ primer, 2μM dNTPs and 1x Moloney murine leukemia virus
158 RT buffer.. The resulting RT products were used for real time PCR amplification.

159

160 **Quantitative Real Time PCR**

161 Total human MCP-1 expression was determined using the primer sequences MCP-1F 5'-
162 GCTCAGCCAGATGCAAT-3'; MCP-1R 5'-GCTTGTCCAGGTGGTCCATG-3'.
163 GAPDH was used as the house keeping gene, using the following primers, GAPDHF 5'-
164 CCACCCATGGCAAAATTCCATG-3'; GAPDHR – 5' TCTAGACGGCAGGTCAGG-
165 3'. 1µl of reverse-transcribed cDNA was subjected to real time PCR using TaKaRa
166 SYBR *Premix Ex Taq* and the Mx3000P quantitative PCR system (Stratagene).Each
167 reaction consisted of 1x SYBR *Premix Ex Taq*, 0.2µM sense and antisense primers, 1µl
168 DNA and H_2O to a final volume of 25µl. Thermal cycler conditions included incubation
169 at 95˚C for 30 seconds followed by 50 cycles of 95˚C for 5 seconds, 60˚C for 30 seconds
170 and 72 ˚C for 15 seconds. Integration of the fluorescent SYBR green into the PCR
171 product was monitored after each annealing step. Amplification of one specific product
172 was confirmed by melting curve analysis.

173

174 **Vectors and Transient Transfections**

175 MCP-1 enhancer and promoter vectors consisted of the pGL3-basic plasmid vector
176 containing either the wild-type human MCP-1 enhancer or promoter regulatory sequences
177 driving a luciferase reporter gene. The enhancer construct harbours two NFκB sites and is
178 contained in the region -2802 to -2573bp relative to the translation start codon. The
179 MCP-1 promoter construct harbours a number of different transcription factor binding
180 sites and contains the proximal section of the wild type human MCP-1 promoter region -
181 161 to -1bp relative to the translation start codon. These constructs have been previously

182 described in detail (16, 25). The NFκB reporter construct, 6NFκBtkluc, contains six
183 copies of the NFκB binding site which is upstream of a minimal thymidine kinase
184 promoter driving a luciferase gene. The NF-κB reporter was a gift from Dr Robert
185 Newton (University of Calgary, Canada).
186
187 FuGene 6 transfection reagent was used to carry out all transient transfections according
188 to the manufacturer's protocol. Human ASM cells were seeded out into 24 well plates at
189 a concentration of $2.5x10^4$ cells/ml in DMEM supplemented media. At 50-60%
190 confluence, cells were growth arrested for 8 hours in serum-free, antibiotic free DMEM
191 containing 4Mm L-glutamine. Fresh serum free antibiotic DMEM containing 4mM L-
192 glutamine was changed after 8 hours and cells were transfected with 0.4µg DNA: 3µl
193 FuGene 6 per well in serum-free, antibiotic free DMEM containing 4mM L-glutamine.
194 After 16 hour transfection, the cells were pretreated for 30 minutes +/- drugs followed by
195 6 hours stimulation with IL-1β (10ng/ml) or TNFα (10ng/ml). Cells were then lysed and
196 firefly and renilla luciferase activity was determined. Data was analysed by dividing the
197 firefly values by the renilla firefly values and expressing the data as the ratio between the
198 two.
199
200 **Western Blot**
201 Western blot was performed to assess the phosphorylation of IkappaB-α in response to
202 TNFα /IL-1β and in the presence of the glucocorticoid ciclesonide. The media was
203 aspirated and cells were washed with ice cold PBS and incubated with phospho-lysis
204 buffer (20mM Tris-HCl Ph 7.4, 1% TritonX-100, 137mM NaCl, 2mM EDTA, 25mM

205 Beta-glycerophosphate, 10% glycerol, 1mM Na3VO4 (sodium orthovanadate), 1mM
206 PMSF, 10μ/ml leupeptin, 1mM DTT, 0.1 U/ml protease inhibitor cocktail). Samples
207 were collected and protein concentration was determined by Bradford assay. The
208 obtained values were converted to protein concentrations (μg/ml) by use of the BSA
209 standard curve. Cell protein (30μg per lane) was subjected to gel electrophoresis on a
210 10% SDS –polyacrylamide gel and electroblotted to PVDF membrane. After blocking
211 (5% milk in TBST), the membrane was incubated overnight at 4 ºC with Phospho IκB-α
212 (Ser 32) (1:1000) or IκB-α (1:1000) primary antibody in 5% Bovine serum albumin
213 (BSA) in 1X TBST with 0.1% Tween20. After washing, the membrane (1x TBST) was
214 incubated with a horseradish peroxidase conjugated secondary antibody (goat-anti-rabbit
215 1:2000 in 5% BSA in 1X TBST with 0.1% Tween20) for 1hour at room temperature. The
216 membrane was incubated with ECL™ Western blotting detection reagent and exposed to
217 Hyperfilm-ECL. Subsequently blots were scanned and densitometry performing using
218 Adobe Photoshop.
219
220 **Statistical Analysis**
221 The mean values of replicate wells for MCP-1 and luciferase levels were calculated and
222 expressed as fold increase. Where MCP-1 absolute values are presented (concentration
223 response experiments), the mean of all the replicates are shown. Each experiment was
224 performed in cells from three different tracheas, each obtained from a different human
225 donor. Three biological replicates were performed per donor cell line. Data were
226 expressed as the mean ± standard error of the mean (SEM). The data from the
227 experiments were subjected to statistical analysis to determine statistical significance

228 using Graphpad Prism version 4.00 for windows (Graphpad Software, San Diegeo,
229 California, USA). One-way analysis of variance of the raw data followed by a Dunnets
230 post test was used to compare all groups to unstimulated control (+). One-way analysis
231 of variance of the raw data followed by a Tukey post test was used to compare stimulated
232 samples to those with drug present (*). A p value of <0.05 was considered statistically
233 significant.
234

235 **Results**

236 **TNFα and IL-1β induce MCP-1 protein release** - We first studied the effect of TNFα
237 and IL-1β on the release of MCP-1 from human ASM cells. Confluent, growth arrested
238 human ASM cells were treated for 24 hours with increasing concentrations of TNFα (up
239 to 100ng/ml) or IL-1β (up to 10ng/ml). TNFα stimulated MCP-1 release in a
240 concentration dependent manner (Figure 1a) above control levels which was significant
241 from concentration 1ng/ml to 100ng/ml. This was also true for IL-1β, with a significant
242 increase in MCP-1 protein from 0.1ng/ml to 10ng/ml of IL-1β stimulation compared to
243 control (Figure 1b).

244

245 **Differential effects of Formoterol and Ciclesonide on IL-1β and TNFα induced**
246 **MCP-1**- Formoterol is a long acting β_2- adrenoceptor agonist (LABA). LABAs are
247 frequently used to treat asthma. Its effects include bronchodilation and inhibition of
248 mediator release from mast cells and monocytes. Serum deprived, confluent Human
249 ASM cells were pre-incubated with the concentrations of Formoterol stated prior to 24
250 hours TNFα/IL-1β stimulation. Formoterol had no effect on TNFα (Figure 2a) or IL-1β
251 (Figure 2b) induced MCP-1 release.

252

253 Glucocorticoids are a first line asthma treatment. Their mechanism of action involves
254 inhibitory effects on pro-inflammatory cytokine production, cell migration and
255 lymphocyte activation. Ciclesonide is an inhaled glucocorticoid with only mild side
256 effects (20). We tested the effect of Ciclesonide on TNFα and IL-1β stimulated MCP-1
257 release from human ASM cells. Serum deprived human ASM cells were pre-treated for

258 30 minutes with a concentration range of Ciclesonide prior to 24 hour TNFα/IL-1β
259 stimulation. Ciclesonide significantly inhibited TNFα stimulated MCP-1 production over
260 the concentration range (10^{-12}-10^{-5}M) (Figure 2c). IL-1β stimulated MCP-1 release was
261 also significantly inhibited in a concentration dependant manner (10^{-12}-10^{-5}M) (Figure
262 2d).
263
264 When Ciclesonide and Formoterol were added in combination, no inhibition further to
265 that elicited by Ciclesonide alone was observed (Figure 2e and 2f).
266
267 **Ciclesonide Inhibits MCP-1 mRNA production and MCP-1 Promoter and Enhancer**
268 **Activity -** To establish whether Ciclesonide was acting transcriptionally to inhibit MCP-1
269 we measured the MCP-1 mRNA level in response to Ciclesonide by quantitative real
270 time PCR. Confluent serum starved human ASM cells were pre-incubated for 30 minutes
271 with 10^{-7}M Ciclesonide. Cells were then stimulated for 8 hours with 0.1ng/ml TNFα or
272 IL-1β. Both TNFα and IL-1β induced MCP-1 mRNA levels (Figure 3a and 3b).
273 Ciclesonide inhibited both TNFα and IL-1β induced MCP-1 (Figure 3a and 3b).
274
275 Subsequently we transfected luciferase reporter constructs for the MCP-1 promoter and
276 enhancer into human ASM to establish whether Ciclesonide affected promoter or
277 enhancer activity. The promoter region construct harbours an NFκB, an Sp1 and an NF-1
278 binding site in addition to two AP-1 sites, while the enhancer region construct harbours 2
279 NFκB binding sites. Human ASM cells were transfected with the MCP-1 promoter
280 reporter for 16 hours and pre-incubated for 30 minutes with 10^{-7}M Ciclesonide before

281 incubating with TNFα (10ng/ml) or IL-1β (10ng/ml) for 2 hours. TNFα and IL-1β
282 significantly increased MCP-1 promoter activity (Figure 3c and 3d). Ciclesonide
283 inhibited MCP-1 promoter activity in the presence of either cytokine.
284
285 Human ASM cells were then transfected with the MCP-1 enhancer reporter. When
286 stimulated with TNFα (10ng/ml) or IL-1β (10ng/ml) MCP-1 enhancer activity was
287 significantly increased compared to control cells and this was inhibited by nearly 2 fold
288 with Ciclesonide (Figure 3e and 3f).
289
290 **IKK-2 Activity is Required for TNFα and IL-1β induced MCP-1 Expression -** As
291 both the MCP-1 promoter and enhancer regions contain NFκB binding sites, we
292 hypothesised that Ciclesonide was acting on the NFκB pathway. Briefly, TNFα and IL-
293 1β signalling can activate IκB kinase 2 (IKK-2). IKK-2 phosphorylates IκB resulting in
294 its degradation. IκB inhibits NfκB signalling when they are associated. Initially we
295 investigated whether TNFα and IL-1β required IKK-2 to stimulate MCP-1 production by
296 incubating cells with TPCA-1. TPCA-1 is an inhibitor of IKK2 and thus inhibits NFκB
297 signalling by preventing IκB degradation. TPCA-1 dose dependently inhibited TNFα
298 (Figure 4a) and IL-1β (Figure 4b) stimulated MCP-1 protein release. A comparable
299 response was observed with a second IKK2 inhibitor, MB120B (Figure 4c and 4d). The
300 same effect was seen with TPCA at the mRNA level (Figure 4e and 4f).
301
302 **Ciclesonide Inhibits Binding of NFκB to its Consensus Sequence -** Having shown that
303 TNFα and IL-1β require IKK2 activity for MCP-1 production we investigated whether

304 Ciclesonide affected the IKK2/IκB/NFκB pathway. Initially we performed western blots
305 to determine whether Ciclesonide was able to prevent IκB degradation. A representative
306 blot is shown in Figure 5a. Both TNFα and IL-1β induced phosphorylation and
307 subsequent degradation of IκB but this was not inhibited in the presence of Ciclesonide
308 (Figure 5b and 5c)
309
310 Finally we established the effect of Ciclesonide on the transcriptional activity of NFκB
311 using a luciferase reporter containing six copies of the NFκB binding site upstream of the
312 luciferase gene. Human ASM cells were transiently transfected with NFκB reporter
313 constructs, pre-treated with Ciclesonide (10^{-7}M) and stimulated with TNFα (10ng/ml) or
314 IL-1β (10ng/ml) for 6 hours. TNFα caused a significant increase (Figure 5d) in NFκB
315 reporter activity, which was inhibited by Ciclesonide. Il-1β also caused a significant
316 increase (Figure 5e) in NFκB reporter activity which was inhibited by Ciclesonide.
317
318 Together these data show that Ciclesonide inhibits TNFα and IL-1β induced MCP-1
319 expression by reducing the potential of NF-κB to associate with its binding site.
320
321 **Discussion**
322 This study is the first to demonstrate that the glucocorticoid Ciclesonide, inhibits TNFα
323 and IL-1β induced MCP-1 expression from human ASM cells via a transcriptional
324 mechanism involving the MCP-1 proximal promoter and distal enhancer and the
325 transcription factor NF-κB.
326

327 Initially we confirmed observations made previously, that MCP-1 is secreted basally
328 from human ASM cells and that TNFα and IL-1β can both concentration dependently
329 increase MCP-1 in the culture supernatant of these cells. We then went on to show that
330 the β_2 agonist Formoterol had no effect on TNFα or IL-1β induced MCP-1 secretion.
331 This is in agreement with previous data from our lab showing Salmeterol has no effect on
332 TNFα induced MCP-1 expression either (16). Contrary to this, cAMP-elevating agents
333 can inhibit MCP-1 secretion from ASM cells (30). However β_2 agonists are also
334 ineffective against IL-1β induced IL-8 secretion from ASM cells while cAMP-elevating
335 agents have an effect, suggesting β_2 agonists act via a different mechanism to direct
336 cAMP elevators (14). We also show that Formoterol has no effect when added in
337 combination with the glucocorticoid Ciclesonide. However, Ciclesonide alone did inhibit
338 both TNFα and IL-1β induced MCP-1 secretion over a concentration range. Ciclesonide
339 also had a small effect on basal MCP-1 expression (data not shown) however the effect
340 on stimulated levels was more dramatic and reproducible. This is the first report of any
341 Ciclesonide effect on human ASM cells. Pype *et al* (21) showed Dexamethasone can
342 inhibit cytomix induced MCP-1 secretion from human ASM cells. They also showed that
343 Dexamethasone reduced cytomix induced MCP-1 mRNA levels suggesting
344 Dexamethasone was acting transcriptionally but no mechanism was established.
345 Conversely, Nie *et al* (16) showed that Fluticasone inhibited TNFα induced MCP-1
346 protein secretion without affecting mRNA levels. Furthermore, Fluticasone did not affect
347 TNFα induced MCP-1 mRNA stability and they concluded that Fluticasone was acting
348 via an undefined posttranscriptional mechanism. While Dhawan *et al* (8) showed PDGF
349 induced MCP-1 secretion from arterial smooth muscle cells is inhibited by

350 Dexamethasone via glucocorticoid receptor dependent destabilisation of MCP-1 mRNA.
351 Hypoxia induced MCP-1 is also inhibited by Dexamethasone in part via effects on
352 mRNA stability (7). IL-1β induced MCP-1 inhibition by any glucocorticoid is not
353 reported. It seems clear that the effects of glucocorticoids on MCP-1 expression are
354 diverse and mediated by mechanisms targeting each step of MCP-1 production.
355
356 To determine whether Ciclesonide was acting transcriptionally in our system we
357 measured MCP-1 mRNA levels in response to TNFα, IL-1β and Ciclesonide. In
358 agreement with previous publications (16, 28, 30), both TNFα and IL-1β induced MCP-1
359 mRNA expression. This was inhibited by Ciclesonide suggesting that Ciclesonide was
360 acting transcriptionally.
361
362 The MCP-1 gene is under the control of a distal enhancer region (-2802 to -2573 relative
363 to the translation start codon) and a proximal promoter region (-161 to -1 relative to the
364 translation start codon). We used luciferase reporters for these regions and determined
365 that both IL-1β and TNFα stimulate both the enhancer and promoter reporters. Both the
366 promoter and enhancer regions reporter activities were inhibited by Ciclesonide. As the
367 enhancer and promoter regions have NF-κB binding sites in common we investigated
368 further the role of upstream NFκB signalling on IL1β and TNFα induced MCP-1
369 expression. In order for NF-κB to translocate to the nucleus and be transcriptionally
370 active it must be released from the inhibitory cytoplasmic protein IκB. Release from IκB
371 requires IκB degradation. IκB kinase (IKK) phosphorylates IκB on two serines residues
372 which mark the protein for ubiquitination and degradation by the proteasome. Thus, we

373 used inhibitors of IKK2, namely TPCA and MB120B, to determine if upstream NFκB
374 signalling was involved in IL-1β and TNFα induced MCP-1 expression. MCP-1 protein
375 secretion was inhibited by both TPCR and MB120B. MCP-1 mRNA levels were reduced
376 in the presence of TPCA. We finally tested whether Ciclesonide could affect either IκB
377 degradation or NFκB binding to its consensus binding sequence. We found that
378 Ciclesonide had no effect on IκB degradation but did inhibit NF-κB binding. This is
379 contrary to a previous report of pancreatitis-associated ascitic fluid (PAAF) induced
380 MCP-1 in pancreatic acinar cells being inhibited by Dexamethasone via prevention of
381 IκB degradation (22). Prevention of IκB degradation prevents NF-κB translocation and
382 subsequently reduces the amount available to bind DNA. Similarly, prednisolone inhibits
383 PDGF stimulated NFκB translocation in human pulmonary artery smooth muscle cells
384 (17). In our system IκB degradation was not changed by Ciclesonide suggesting the
385 change in NFκB reporter activity is due to modulation of NFκB binding as opposed to
386 changes in nuclear NFκB levels. Interestingly Amrani *et al* (1) have shown that
387 Dexamethasone cannot inhibit TNFα induced activation of the NFκB reporter in airway
388 smooth muscle cells, suggesting different glucocorticoids may modulate TNFα signalling
389 differently. Furthermore, Park *et al* (19) showed Dexamethasone inhibition of TNFα
390 induced MCP-1 expression from human glomerular endothelial cells was independent of
391 IκB degradation, and NFκB binding. Studies investigating genes other than MCP-1 have,
392 however, shown similar affects to those seen in the current study. For example,
393 inhibition of TNFα induced IL-1β expression by Dexamethasone in rheumatoid arthritis
394 synovial cells occurs via a change in NFκB binding ability that is not dependent on IκB
395 degradation or a change in nuclear NFκB p65 levels (11). There are reports of

396 glucocorticoid mediated inhibition of NF-κB Serine276 phosphorylation and Histone H3
397 serine 10 phosphorylation (2). Abolishment of NF-κB S276 phosphorylation prevents its
398 binding to DNA (23) and phosphorylated H3 serine 10 is associated with open chromatin
399 and active transcription and is suggested as a marker for NFκB recruitment (5). It is
400 possible that Ciclesonide is inhibiting upstream kinase signalling to modify NFκB or the
401 chromatin environment in order to regulate MCP-1 expression, however in data not
402 shown we failed to see any effect of Ciclesonide on NF-κB phospho S536 and were
403 unable to detect a signal for NF-κB phosphor S276.
404
405 In conclusion, we have made the novel observation that inhibition of TNFα and IL-1β
406 induced MCP-1 expression from human ASM cells by the glucocorticoid Ciclesonide is
407 mediated via a transcriptional mechanism that it not dependent on IκB degradation, but
408 on the ability of TNFα and IL-1β induced NFκB p65 to bind its consensus binding site.
409 The effect of glucocorticoids on both MCP-1 expression and NF-κB signalling is diverse
410 and this study provides new insight into a complex field.
411

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416

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518
519

520 **Figure Legends**

521 **Figure 1**. TNFα and IL-1β induce MCP-1 secretion. *A,* Human ASM cells were treated
522 for 24 hours with TNFα (0-100ng/ml) and MCP-1 secretion was measured by ELISA. *B*,
523 Human ASM cells were treated for 24 hours with IL-β (0-10ng/ml) and MCP-1 secretion
524 was measured by ELISA. Each bar represents mean ± SEM (n=3). ++p<0.01 compared
525 to control.

526

527 **Figure 2.** Differential effects of Ciclesonide and Formoterol on TNFα or IL-1β induced
528 MCP-1 secretion. Human ASM cells were pre-treated with Formoterol (10^{-7}-10^{-6}M) for
529 30 minutes prior to 24 hour *(A)* TNFα (0.1ng/ml) stimulation and (*B*) IL-1β (0.1ng/ml)
530 stimulation. The MCP-1 released into the culture medium was measured by ELISA.
531 Each point represents mean ± SEM (n=4). Human ASM cells were pre-incubated for 30
532 minutes with Ciclesonide (10^{-12}-10^{-5}M) and then stimulated for 24 hours with (*C*) TNFα
533 (0.1ng/ml) and (*D*) IL-1β (0.1ng/ml). The MCP-1 released into the culture medium was
534 measured by ELISA. Each point represents mean ± SEM (n=3). Human ASM cells were
535 pre-treated with Formoterol (10^{-7}M) or Ciclesonide (10^{-8}M) or both 30 minutes prior to
536 24 hour (*E*) TNFα stimulation (0.1ng/ml) and (*F*) IL-1β stimulation (0.1ng/ml). The
537 MCP-1 released into the culture medium was measured by ELISA. Each point

538 represents mean ± SEM (n=3). $^{+++}$P<0.001 compared to control, *P<0.05,
539 **P<0.01***P<0.001 compared to stimulated cells.
540
541 **Figure 3.** Ciclesonide inhibits MCP-1 mRNA production and MCP-1 promoter and
542 enhancer activity. Human ASM cells were pre-incubated for 30 minutes with 10^{-7}M
543 Ciclesonide prior to 8 hour (*A*) TNFα (0.1ng/ml) stimulation and (*B*) IL-1β (0.1ng/ml)
544 stimulation. Cells were lysed, RNA extracted, reverse transcribed and cDNA levels
545 determined by qPCR. Each point represents mean ± SEM (n=3). 50-60% confluent
546 Human ASM cells were transfected with 0.4μg/well MCP-1 promoter plasmid for 16
547 hours. The transfected cells were pre-treated with Ciclesonide (10^{-7}M) for 30 minutes
548 then incubated with (*C*) TNFα (10ng/ml) for 6 hours and (*D*) IL-1β (10ng/ml) for 6
549 hours. The firefly and renilla luciferase activity were assayed and a ratio calculated to
550 represent the activities of the reporter. Each point represents mean ± SEM (n=3). 50-60%
551 confluent Human ASM cells were transfected with 0.4μg/well MCP-1 enhancer plasmid
552 for 16 hours. The transfected cells were pre-treated with Ciclesonide (10^{-7}M) for 30
553 minutes then incubated with (*E*) TNFα (10ng/ml) for 6 hours and (*F*) IL-1β (10ng/ml) for
554 6 hours. The firefly and renilla luciferase activity were assayed and a ratio calculated to
555 represent the activities of the reporter. Each point represents mean ± SEM (n=3).
556 $^{+}$P<0.05, $^{+++}$P<0.001 compared to control, *P<0.05, ** P<0.01 compared to stimulated
557 alone.
558
559 **Figure 4.** IKK-2 activity is required for TNFα and IL1β induced MCP-1 secretion.
560 Human ASM cells were pre-treated with TPCA-1 (*A and B*) or MB120B (*C and D*) for

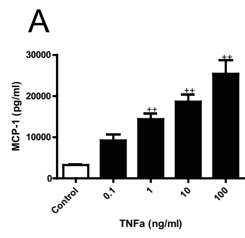
561 30 minutes and then incubated for 24 hours with (*A and C*) TNFα (0.1ng/ml) and (*B and*
562 *D*) IL-1β (0.1ng/ml). Each bar represents mean ± SEM (n=3). Human ASM cells were
563 pre-incubated for 30 minutes with 10^{-5}M TPCA prior to 8 hour (*E*) TNFα (0.1ng/ml)
564 stimulation and (*F*) IL-1β (0.1ng/ml) stimulation. Cells were lysed, RNA extracted,
565 reverse transcribed and cDNA levels determined by qPCR. Each bar represents mean ±
566 SEM (n=3). $^{+}P<0.05$, $^{+++}P<0.001$, compared to control. *p<0.05, **p<0.01, ***p<0.001,
567 compared to stimulated alone.

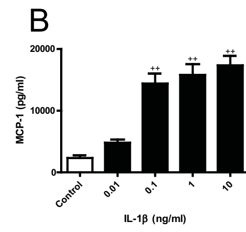
568

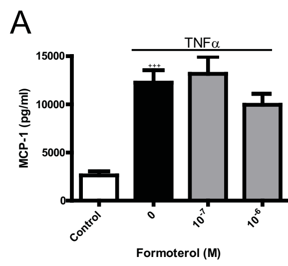
569 **Figure 5.** Ciclesonide does not affect IκB degradation but does inhibit NFκB binding to
570 its consensus sequence. *A,* Human ASM cells were pre-incubated with Ciclesonide for
571 30 minutes then incubated with or without TNFα (0.1ng/ml) or IL-1β (0.1ng/ml) for 5
572 and 15 minutes. Cells were lysed and run on a western blot. The membrane was probed
573 for phospho and total IκB. A representative blot is shown of 3 independent experiments
574 in 3 donor cell lines. Western blots for phospho and total IκB were scanned and
575 densitometry performed for (*B*) TNFα and (*C*) IL-1β stimulated samples. Each point
576 represents mean ± SEM (n=3). 50-60% confluent Human ASM cells were transfected
577 with 0.4μg/well NFκB reporter plasmid for 16 hours. The transfected cells were pre-
578 treated with Ciclesonide (10^{-7}M) for 30 minutes then incubated with (*D*) TNFα (10ng/ml)
579 for 6 hours and (*E*) IL-1β (10ng/ml). The firefly and renilla luciferase activity were
580 assayed and a ratio calculated to represent the activities of the reporter. Each point
581 represents mean ± SEM (n=3). $^{++}p<0.01$, $^{+++}P<0.001$ compared to control, *p<0.05
582 compared to stimulated alone.

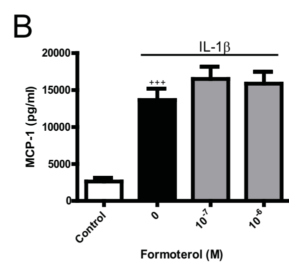
583

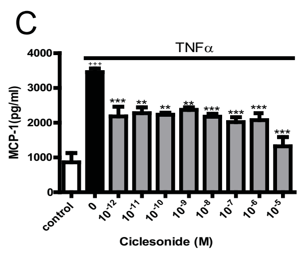
584

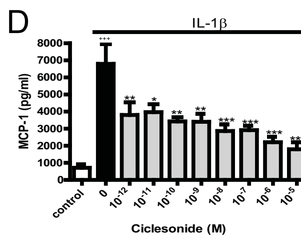

A
30000
20000
10000
0
MCP-1 (pg/ml)
++
++
++
Control
0.1
1
10
100
TNFa (ng/ml)

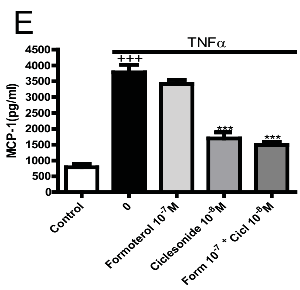

B
20000
10000
0
MCP-1 (pg/ml)
++
++
++
Control
0.01
0.1
1
10
IL-1β (ng/ml)

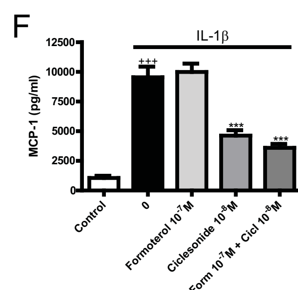

A
TNFα
MCP-1 (pg/ml)
15000
10000
5000
0
+++
Control
0
10^{-7}
10^{-6}
Formoterol (M)


B
IL-1β
MCP-1 (pg/ml)
20000
15000
10000
5000
0
+++
Control
0
10^{-7}
10^{-6}
Formoterol (M)

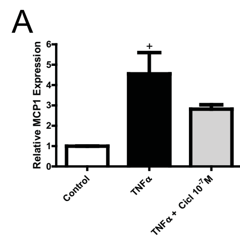

C
TNFα
MCP-1(pg/ml)
4000
3000
2000
1000
0
+++
***
**
**
**
***
***
***
***
control
0
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Ciclesonide (M)


D
IL-1β
MCP-1 (pg/ml)
8000
7000
6000
5000
4000
3000
2000
1000
0
+++
**
*
**
**
***
***
***
***
control
0
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Ciclesonide (M)


E
TNFα
MCP-1(pg/ml)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
+++
***
***
Control
0
Formoterol 10^{-7}M
Ciclesonide 10^{-8}M
Form 10^{-7} + Cicl 10^{-8}M


F
IL-1β
MCP-1 (pg/ml)
12500
10000
7500
5000
2500
0
+++
***
***
Control
0
Formoterol 10^{-7}M
Ciclesonide 10^{-8}M
Form 10^{-7}M + Cicl 10^{-8}M

A



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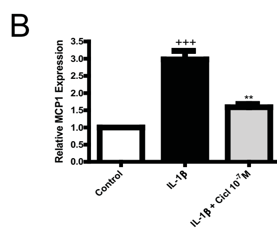

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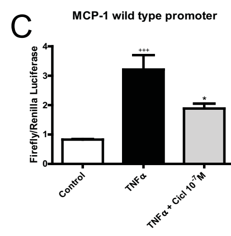
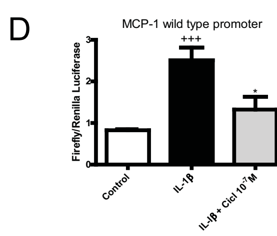


D



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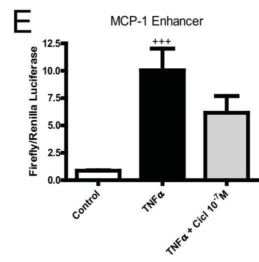

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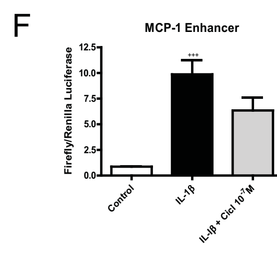
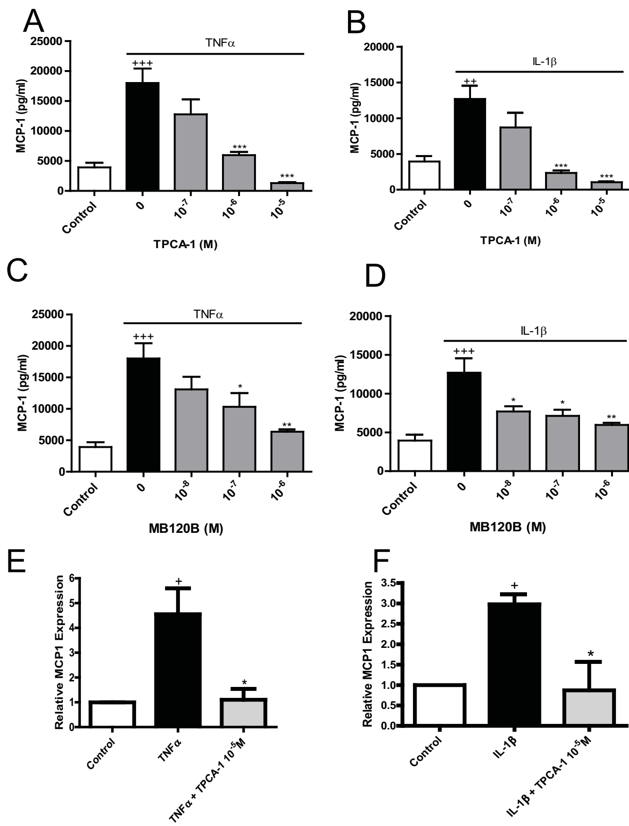
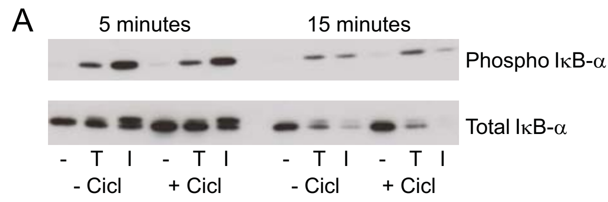
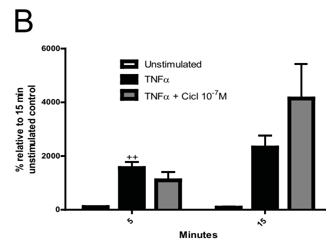
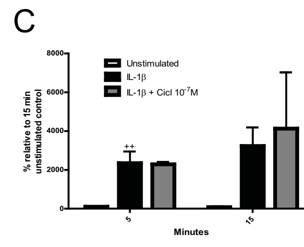
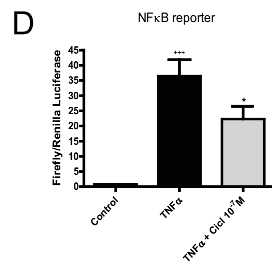


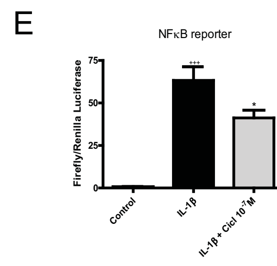

A
TNFα
MCP-1 (pg/ml)
TPCA-1 (M)
B
IL-1β
MCP-1 (pg/ml)
TPCA-1 (M)
C
TNFα
MCP-1 (pg/ml)
MB120B (M)
D
IL-1β
MCP-1 (pg/ml)
MB120B (M)
E
Relative MCP1 Expression
Control
TNFα
TNFα + TPCA-1 10⁻⁵M
F
Relative MCP1 Expression
Control
IL-1β
IL-1β + TPCA-1 10⁻⁵M


A
5 minutes
15 minutes
Phospho IκB-α
Total IκB-α
- T I - T I - T I - T I
- Cicl
+ Cicl
- Cicl
+ Cicl


B
Unstimulated
TNFα
TNFα + Cicl 10⁻⁷M
% relative to 15 min unstimulated control
6000
4000
2000
0
++
5
15
Minutes


C
Unstimulated
IL-1β
IL-1β + Cicl 10⁻⁷M
% relative to 15 min unstimulated control
8000
6000
4000
2000
0
++
5
15
Minutes


D
NFκB reporter
Firefly/Renilla Luciferase
45
40
35
30
25
20
15
10
5
0
***
*
Control
TNFα
TNFα + Cicl 10⁻⁷M


E
NFκB reporter
Firefly/Renilla Luciferase
75
50
25
0
***
*
Control
IL-1β
IL-1β + Cicl 10⁻⁷M