Down-regulation of Cytokine-induced Interleukin-8 Requires Inhibition of p38 Mitogen-activated Protein Kinase (MAPK) via MAPK Phosphatase 1-dependent and -independent Mechanisms**

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Down-regulation of overabundant interleukin (IL)-8 present in cystic fibrosis (CF) airways could ease excessive neutrophil burden and its deleterious consequences for the lung. IL-8 production in airway epithelial cells, stimulated with e.g. inflammatory cytokines IL-1β and tumor necrosis factor (TNF)-α, is regulated by several signaling pathways including nuclear factor (NF)-κB and p38 mitogen-activated protein kinase (MAPK). We previously demonstrated that the anti-inflammatory drugs dexamethasone and ibuprofen suppress NF-κB; however, only dexamethasone down-regulates cytokine-induced IL-8, highlighting the importance of non-NF-κB mechanisms. Here, we tested the hypothesis that down-regulation of cytokine-induced IL-8 requires modulation of the MAPK phosphatase (MKP)-1/p38 MAPK/mRNA stability pathway. The effects of dexamethasone (5 nM) and ibuprofen (480 μM) on this pathway and IL-8 were studied in CF (CFTE29o–, CFBFE41o–) and non-CF (1HAEo–) airway epithelial cells. We observed that dexamethasone, but not ibuprofen, destabilizes IL-8 mRNA and up-regulates MKP-1 mRNA. Further, siRNA silencing of MKP-1, via p38 MAPK, leads to IL-8 overproduction and diminishes the anti-IL-8 potential of dexamethasone. However, MKP-1 overexpression does not significantly alter IL-8 production. By contrast, direct inhibition of p38 MAPK (inhibitor SB203580) efficiently suppresses IL-8 with potency comparable with dexamethasone. Similar to dexamethasone, SB203580 decreases IL-8 mRNA stability. Dexamethasone does not affect p38 MAPK activation, which excludes its effects upstream of p38 MAPK. In conclusion, normal levels of MKP-1 are necessary for a full anti-IL-8 potential of pharmacological agents; however, efficient pharmacological down-regulation of cytokine-induced IL-8 also requires direct effects on p38 MAPK and mRNA stability independently of MKP-1.

Inflammation is an integral part of the immune response of the body and is, normally, self-limiting. In many inflammatory lung diseases, including cystic fibrosis (CF),3 inflammation is dysregulated and cannot curb itself and, therefore, requires pharmacological interventions. Specifically in CF, excessive airway inflammation leads to respiratory failure and premature demise. Systemic corticosteroids have effectively suppressed inflammation in CF and slowed progression of lung disease (1–3), unfortunately, at a cost of extensive adverse effects, such as growth retardation and diabetes (4, 5). As an alternative to corticosteroids, high doses of ibuprofen have been successfully tried in CF (6, 7), but its clinical use is also limited by adverse effects (8–10).

The insufficient safety of current anti-inflammatory therapies necessitates the search for more targeted therapies. A better understanding of the anti-inflammatory mechanisms of existing drugs could help to uncover intracellular targets to develop novel, targeted, and safer therapies.

Interleukin (IL)-8 is the major neutrophil-attracting chemokine (11) present at high levels in airways of patients with CF (12–23). These high levels are likely to be responsible for abundant neutrophils in airway secretions of these patients (13, 14, 19, 21, 24, 25). Down-regulation of IL-8, alone or in combination with other cytokines, suppresses neutrophil chemotaxis and activation (22, 26), and could, therefore, ease the neutrophil burden in CF airways.

Airway epithelium is one of the major producers of IL-8. IL-8 is normally secreted at negligible amounts and is greatly up-regulated upon e.g. exposure to IL-1β or tumor necrosis factor (TNF)-α, the early response cytokines (27). Cytokine-induced IL-8 is controlled by transcription factors and mitogen-activated protein kinases (MAPKs) (28, 29). The major transcription factor is nuclear factor (NF)-κB (28, 29). MAPKs include p38 MAPK, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). In the IL-8 signaling cascade, MAPKs work as upstream activators or enhancers of the aforementioned transcription factors. In addition, p38 MAPK stabi-

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3 The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; MKP-1, MAPK phosphatase-1; MAP2K, mitogen-activated protein kinase kinase; qPCR, quantitative PCR; DMSO, dimethyl sulfoxide.
lizes IL-8 mRNA and, thus, directly potentiates IL-8 production independently of transcription factors.

Corticosteroids are known to down-regulate IL-8 production in airway epithelium exposed to a variety of stimuli (30–36). The anti-IL-8 mechanisms of corticosteroids are still poorly understood. Various mechanisms, from suppression of NF-κB to inhibition of p38 MAPK, can be responsible for the anti-IL-8 effects of corticosteroids. Our previous study highlighted the importance of non-NF-κB mechanisms in pharmacological down-regulation of cytokine-induced IL-8 (37). Specifically, we observed that clinical doses of ibuprofen and the corticosteroid dexamethasone (400 μM and 5 nM, respectively) suppress NF-κB, yet only dexamethasone down-regulates IL-8 (37).

Besides NF-κB, the anti-IL-8 mechanisms of corticosteroids can be linked to MAPK phosphatases (MKPs) (38, 39) and, specifically, MKP-1 (40–42). MKP-1 is a negative regulator of p38 MAPK and is transcriptionally up-regulated by corticosteroids. Through the MKP-1/p38 MAPK pathway, corticosteroids could potentially control two signaling cascades pertinent to IL-8: (i) the p38 MAPK/NF-κB (43) and (ii) the p38 MAPK/mRNA stability cascade (42). Because inhibition of NF-κB transactivation is not specifically associated with pharmacological down-regulation of cytokine-induced IL-8 (37), in the present study, we tested the hypothesis that dexamethasone down-regulates IL-8 by affecting the MKP-1/p38 MAPK/mRNA stability pathway and that ibuprofen lacks this ability and is, hence, ineffective against cytokine-induced IL-8. Our study provides evidence that normal levels of MKP-1 are, indeed, required for a full anti-IL-8 potential of pharmacological down-regulation of IL-8 (37), in the present study, we tested the hypothesis that dexamethasone down-regulates IL-8 by affecting the MKP-1/p38 MAPK/mRNA stability pathway and that ibuprofen lacks this ability and is, hence, ineffective against cytokine-induced IL-8. Our study provides evidence that normal levels of MKP-1 are, indeed, required for a full anti-IL-8 potential of pharmacological agents such as dexamethasone. However, our findings further indicate that efficient pharmacological down-regulation of cytokine-induced IL-8 requires direct effects on p38 MAPK and mRNA stability independently of MKP-1.

EXPERIMENTAL PROCEDURES

Reagents—Cell culture flasks were from Nunc Nalgene or Greiner; multiwell plates and culture dishes were from Nunc Nalgene. Transwell permeable supports (6.5-mm diameter, 0.4-μm pore size) were from Corning. (+)-Ibuprofen was from Cayman Chemicals, and dexamethasone was from Sigma. Both drugs were prepared as before (37), and ethanol was used as a diluent control. Human recombinant IL-1β and TNF-α were from BD Biosciences. Unless otherwise specified, reagents were obtained from Sigma.

Cell Culture—Cells were maintained as described previously (37). As in our previous study (37), we conducted the majority of experiments with CFTE29o− (homozygous for ΔF508 cystic fibrosis transmembrane regulator [CFTR] (44)) and 1HAEo− (wild-type CFTR (37)) cell lines grown under submerged conditions. Key experiments were confirmed in CFBE41o− cell line (homozygous for ΔF508 mutated CFTR (37)), which forms tight junctions and differentiates on air-liquid interface (45–47). Cell lines were a kind gift from Dr. Dieter Gruenert (University of California, San Francisco, CA). Exponentially growing cells were trypsinized (37), counted, and used in experiments as described below.

Cell Stimulation—Cell stimulation was done using 10 ng/ml IL-1β or TNF-α (37). Briefly, intact or transfected cells were plated onto multiwell plates, cultured to 95–100% confluency, and preconditioned for 0.5 h in antibiotic-free minimal essential medium supplemented with 0.5% bovine serum albumin and 2 mM l-glutamine. Preincubation with test drugs also occurred at this step. Then, cells were stimulated with inflammatory stimuli and collected for experimental outcomes as described below. Alternatively, 0.5 × 10^5 CFBE41o− cells were plated onto precoated (37) Transwell permeable supports. After 24 h, cells became confluent. Apical culture medium was removed, and cells were grown on air-liquid interface for 14 days. Culture medium (37) was changed in the basolateral compartment every 48 h. CFBE41o− cells grown under these conditions demonstrated a transepithelial resistance of >250 ohms × cm² (data not shown). Prior to stimulation, basal medium was changed to antibiotic-free minimal essential medium supplemented with 0.5% bovine serum albumin and 2 mM l-glutamine, with or without added test drugs. After preconditioning for 0.5 h, IL-1β was added to basolateral medium.

IL-8 ELISA—Cells grown on 24-well plates or Transwell permeable supports were stimulated, and culture supernatants were collected and analyzed for IL-8 by ELISA (BD Biosciences) (37).

IL-8 and MKP-1 mRNA Expression—Cells grown on 12-well plates were lysed with TRIzol (Invitrogen). Total RNA was extracted, reverse-transcribed, and used in qPCR (37). Primer sets (IL-8, MKP-1, and β-actin) were from Qiagen. IL-8 and MKP-1 mRNAs were normalized to β-actin mRNA and expressed as fold change over basal/control conditions (37).

Transient Transfection—For high efficiency transient transfection, we used the Nucleofector II device (Lonza) as described previously (37). The transfection protocols were optimized for each cell line, and transfection efficiencies were always >80% (data not shown). For siRNA transfections of CFTE29o− and 1HAEo− cells, we used Nucleofector solutions V and L (Lonza) and Nucleofector programs T-020 and T-030, respectively. CFBE41o− cells were transfected in Nucleofector solution V using the program X-005. For the MKP-1 overexpression experiments in CFTE29o− cells, we used Ingenio electroporation solution (Mirus Bio).

MKP-1 siRNA—Two million cells were transfected in the presence of 200, 20, or 2 pmol of either Silencer negative control #1 or MKP-1 siRNA (s4363; both from Ambion). Cell concentration was adjusted to 0.5 × 10^6 cells/ml, and cells were plated onto multiwell dishes and cultured for 48 h. Then, cells were collected for MKP-1 mRNA expression analysis (qPCR) to confirm efficiency of MKP-1 silencing. Parallel cells were used in experiments as described under “Results.”

MKP-1 Overexpression—pFlag-CMV2-MKP-1 plasmid was a kind gift of Dr. Andrew R. Clark (Imperial College, London, UK) (42). One million cells were transfected in the presence of 100 or 500 ng of either control plasmid or pFlag-CMV2-MKP-1. Cells were cultured for 24 h and collected for MKP-1 mRNA expression analysis (qPCR) to confirm the efficiency of MKP-1 overexpression. Parallel cells were used in experiments as described under “Results.”
MKP-1 and p38 MAPK in Pharmacological Inhibition of IL-8

A. test drugs

IL-8 mRNA

IL-1β for 1 hr
after IL-1β, 2 hrs

B. test drugs

IL-8 mRNA

0.5
1.0
1.0
1.5
2.0

with IL-1β, hrs
after IL-1β, hrs

C. test drugs

IL-8 mRNA

IL-1β for 1 hr
after IL-1β, 2 hrs
ActD for 1.5 hrs

D. test drugs

IL-8 mRNA

0
10
20
30
40
50
60
70
80
90
100

with ActD, hrs

FIGURE 1. Effects of test drugs on kinetics and stability of IL-8 mRNA in CFTE290 cells. A, CFTE290 cells were preincubated for 0.5 h with test drugs (diluent; 480 μM ibuprofen; or 5 nM dexamethasone) and stimulated for 1 h with 10 ng/ml IL-1β. After removal of IL-1β, cells were incubated for up to 2 h with test drugs. B, at the indicated times, cells were collected to quantify IL-8 mRNA levels by qPCR. *, p < 0.05 versus diluent (dil), ibu, 480 μM ibuprofen; dex, 5 nM dexamethasone. C, parallel cells were stimulated for 1 h with 10 ng/ml IL-1β. After removal of IL-1β, cells were incubated for 2 h with test drugs and subsequently exposed to actinomycin D (ActD, 5 μg/ml) for 0–1.5 h. D, at the indicated times, cells were collected to quantify IL-8 mRNA levels by qPCR. The mRNA half-life was calculated using the one-phase decay analysis in GraphPad Prism software. In A and C, the down- and upwards arrows, respectively, represent time points for intervention and obtaining of sample, and the rightwards arrows indicate the length of intervention. Data are means ± S.E.

**MAPK Inhibition**—p38 MAPK inhibitor SB203580 and control inhibitor SB202474 (both from EMD Biosciences) were used in the majority of experiments. In selected experiments, we used JNK inhibitor SP600125 and mitogen-activated protein kinase kinase (MAP2K) inhibitor U0126 (EMD Biosciences). MAPK inhibitors were dissolved in anhydrous DMSO at a concentration of 10 mM and stored at −20°C. For experiments, the inhibitors were diluted in culture medium at the desired concentrations; the concentration of DMSO never exceeded 0.01%.

**p38 MAPK Activation**—Cells grown onto 35-mm culture dishes were stimulated and collected in cell lysis buffer (Cell Signaling) supplemented with protease and phosphatase inhibitor mixture. Protein concentrations were quantified and normalized using BCA assay (Pierce). Activation (i.e. phosphorylation) of p38 MAPK was assessed using PathScan ELISA (Cell Signaling).

**mRNA Decay**—IL-8 mRNA decay was assessed in actinomycin D pulse-chase experiments as described previously (48). In brief, submerged cells grown on 12-well plates were stimulated, incubated with 5 μg/ml actinomycin D (a transcriptional inhibitor) for 0–1.5 h, and lysed with TRIzol. Total RNA was extracted, reverse-transcribed, and used in qPCR (37). Well differentiated CFBE41o− cells grown on Transwell permeable supports were stimulated, incubated with actinomycin D added to basolateral culture medium, and collected in lysis buffer RLT (QiaGen). Total RNA was extracted using an RNeasy micro kit (QiaGen), reverse-transcribed, and used in qPCR (37). IL-8 mRNA levels were normalized to β-actin mRNA (37). The 1.5-h incubation with actinomycin D did not affect expression of β-actin mRNA (data not shown).

**Statistical Analysis**—At least three independent experiments were done for each study. Most statistical analyses were conducted with the help of Statistica 6.0 software (StatSoft). mRNA decay was assessed using the one-phase decay analysis in GraphPad Prism (GraphPad Software). The IC50 (i.e. the concentration that caused a 50% inhibition of IL-8 production) values of the test drugs were calculated using the nonlinear regression analysis in GraphPad Prism. The results are presented as means ± S.E. Differences between groups were assessed by the Mann-Whitney U test or repeated measures analysis of variance, where appropriate. The level of statistical confidence was set at p < 0.05. Statistical significances are presented as * (p < 0.05) or *** (p < 0.001).

**RESULTS**

**Effects of Test Drugs on IL-8 mRNA Kinetics and Stability**—Pharmacological agents down-regulate IL-8 via transcriptional and/or post-transcriptional mechanisms. Here, we evaluated the effects of clinical doses of ibuprofen and dexamethasone (480 μM and 5 nM, respectively) on mRNA kinetics and stability of cytokine-stimulated IL-8.

We first examined IL-8 mRNA kinetics in CFTE290 cells pretreated for 0.5 h with ibuprofen or dexamethasone and stimulated with 10 ng/ml IL-1β. IL-8 mRNA levels increased steadily during stimulation (Fig. 1B), and neither test drug affected these IL-8 mRNA increases in the presence of IL-1β (Fig. 1B). Further, ibuprofen did not alter kinetics of IL-8
mRNA after withdrawal of IL-1β (Fig. 1B). By contrast, dexamethasone steadily down-regulated IL-8 mRNA after IL-1β was withdrawn (Fig. 1B).

We further tested the effects of test drugs on IL-8 mRNA stability. CFTE290− cells, pretreated with ibuprofen or dexamethasone and stimulated with IL-1β, were subjected to actinomycin D pulse-chase. As expected, ibuprofen did not affect IL-8 mRNA stability (Fig. 1D), whereas dexamethasone potently decreased the half-life of IL-8 mRNA (Fig. 1D). A similar IL-8 mRNA decay in the presence of dexamethasone was observed in 1HAEo− cells (data not shown). These results indicate that dexamethasone, but not ibuprofen, facilitates decay of IL-8 mRNA. In the next set of experiments, we studied the involvement of MKP-1 in the decay of IL-8 mRNA induced by dexamethasone.

**Effects of Test Drugs on MKP-1 Expression**—Studies by others (41–43, 49–51) demonstrated that corticosteroids transcriptionally up-regulate MKP-1. There is no published data on whether ibuprofen modulates MKP-1 expression. In the next experiment, we examined the effects of test drugs on MKP-1 mRNA levels in intact (i.e. nonstimulated) and stimulated CFTE290− cells.

In line with previous studies, we observed that dexamethasone increased levels of MKP-1 mRNA in nonstimulated cells (Fig. 2B). The magnitude of this up-regulation (~2.5-fold) was comparable with previously published data. Importantly, ibuprofen did not affect MKP-1 mRNA expression.

Stimulation with IL-1β increased MKP-1 mRNA expression at ~2-fold (Fig. 2D). Dexamethasone, but not ibuprofen, tended to potentiate the increase in MKP-1 mRNA caused by IL-1β (Fig. 2D). These experiments demonstrate that MKP-1 is transcriptionally up-regulated by inflammatory stimuli and dexamethasone, but not by ibuprofen.

**Effects of MKP-1 Overexpression on Down-regulation of IL-8**—Having observed that expression of MKP-1 mRNA is modestly up-regulated by dexamethasone, we asked whether this up-regulation is the principal mechanism to down-regulate cytokine-induced IL-8. Here, we evaluated how increases in MKP-1 expression down-regulate IL-8. CFTE290− cells were transiently transfected with control or MKP-1 plasmids. As expected, control plasmid did not alter MKP-1 mRNA levels, whereas there was a substantial increase in MKP-1 mRNA after transfection with MKP-1 plasmid (Fig. 3B).

In subsequent experiments, we stimulated control or MKP-1 overexpressing cells with IL-1β to evaluate the IL-8 down-regulating ability of MKP-1 overexpression. We observed that even these highly elevated (i.e. >100-fold) levels of MKP-1 exhibited only a trend toward down-regulation of cytokine-induced IL-8 (Fig. 3D).

We next tested whether overexpressed MKP-1, similar to dexamethasone, modulates IL-8 mRNA stability. Cells overexpressing control plasmid or MKP-1 were subjected to stimulation with IL-1β and actinomycin D pulse-chase. MKP-1 overexpression, even at >100-fold over control cells, did not alter IL-8 mRNA stability (data not shown). These results indicate that MKP-1 overexpression only leads to modest decreases in cytokine-induced IL-8 because it lacks the ability to destabilize IL-8 mRNA.

**MKP-1 Silencing Potentiates Cytokine-induced IL-8 and Diminishes the Anti-IL-8 Effects of Dexamethasone**—After we observed that MKP-1 overexpression did not lead to increased IL-8 mRNA decay, we next asked to what extent physiological...
levels of MKP-1 determine the mRNA destabilizing effects of dexamethasone. First, we evaluated how MKP-1 silencing determines the mRNA destabilizing effects of dexamethasone. We, therefore, wished to functionalize the IL-8 overproduction in MKP-1 silenced cells, we transfected control or MKP-1 siRNA, pretreated these cells with dexamethasone, and subjected them to actinomycin D pulse-chase. Treatment with dexamethasone destabilized IL-8 mRNA in both control and MKP-1 silenced cells (Fig. 6B). Further, MKP-1 silencing did not alter the ability of dexamethasone to destabilize IL-8 mRNA.

**The Steady-state Levels of IL-8 mRNA Are Increased in MKP-1 Silenced Cells but Not Due to Increased mRNA Stability**—We transfected CFTE29o− cells with control or MKP-1 siRNA, pretreated these cells with dexamethasone, stimulated them with IL-1β, and subjected them to actinomycin D pulse-chase. Treatment with dexamethasone destabilized IL-8 mRNA in both control and MKP-1 silenced cells (Fig. 6B). Further, MKP-1 silencing did not alter the ability of dexamethasone to destabilize IL-8 mRNA.

MKP-1 Silencing Does Not Affect the Destabilization of IL-8 mRNA by Dexamethasone—We transfected CFTE29o− cells with control or MKP-1 siRNA, pretreated these cells with dexamethasone, stimulated them with IL-1β, and subjected them to actinomycin D pulse-chase. Treatment with dexamethasone destabilized IL-8 mRNA in both control and MKP-1 silenced cells (Fig. 6B). Further, MKP-1 silencing did not alter the ability of dexamethasone to destabilize IL-8 mRNA. The above reported findings indicate that physiological levels of MKP-1 are essential for dexamethasone to exert its maximal anti-IL-8 effects. Subsequent experiments were done to test whether MKP-1 silencing alters the anti-IL-8 effects of dexamethasone via diminishing its ability to destabilize IL-8 mRNA.

**The Role of p38 MAPK in IL-8 Overproduction in MKP-1 Silenced Cells**—MKP-1 exerts its anti-inflammatory effects by deactivating MAPKs. Its effects on p38 MAPK could potentially control both transcriptional and post-transcriptional (i.e. mRNA stability) pathways of IL-8 regulation. We, therefore, examined the involvement of p38 MAPK in IL-8 overproduction observed in intact or MKP-1 silenced cells.

In cells with intact MKP-1, stimulation with IL-1β resulted in a significant (p < 0.05) activation of p38 MAPK, which peaked within the first minutes of stimulation (Fig. 7B). It has been reported previously (43) that MKP-1 silencing further potentiates activation of p38 MAPK. We, therefore, wished to func-
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Although p38 MAPK and JNK are preferential substrates for MKP-1 (38, 39, 52), this phosphatase may also deactivate ERK (38, 39, 52). Therefore, we next ruled out the involvement of ERK in IL-8 hyperproduction in MKP-1 silenced cells. As there are no specific ERK inhibitors, we utilized the inhibitor U0126, which inactivates MAP2K, the upstream activator of ERK. As expected, ERK was not involved in hyperproduction of IL-8 in MKP-1 silenced cells (supplemental Fig. S4E). These experiments indicate that p38 MAPK, but not JNK or ERK, is involved in the overproduction of IL-8 in cytokine-stimulated cells with silenced MKP-1.

Effects of Inhibition of p38 MAPK on Cytokine-induced IL-8—

In the experiments described above, we observed that dexamethasone destabilizes IL-8 mRNA, whereas modulation of MKP-1 levels has no effect on IL-8 mRNA stability. p38 MAPK is known to control mRNA stability (28, 29). We next asked whether dexamethasone exerts its effects directly (i.e. bypassing MKP-1) on p38 MAPK. If that is the case, we argued, then a direct inhibition of p38 MAPK should recapitulate the anti-IL-8 effects of dexamethasone.

We then evaluated whether a direct inhibition of p38 MAPK would efficiently down-regulate cytokine-induced IL-8 and destabilize IL-8 mRNA, both being characteristic features of dexamethasone. We observed that inhibition of p38 MAPK by SB203580 significantly and dose-dependently down-regulated IL-8 in cytokine-stimulated cells and that its anti-IL-8 potency was comparable with dexamethasone (IC_{50} of 8.4 and 2.4 nM, respectively; Fig. 8B). We further observed that SB203580, similar to dexamethasone, facilitated IL-8 mRNA decay (Fig. 8D). Therefore, our findings indicate that the MKP-1-independent inhibition of p38 MAPK recapitulates the anti-IL-8 effects of dexamethasone.
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By contrast, inhibition of either JNK or ERK did not affect IL-8 production in CFTE29o cells with intact or silenced MKP-1. A, CFTE29o cells were pretreated for 1 h with 10 ng/ml IL-1β. B, at 0, 5, or 60 min of stimulation, cells were collected for analysis of p38 MAPK activation by PathScan ELLISA. The p38 MAPK activation is presented as the percentage of that under basal conditions (i.e. 0 min of stimulation). *, p < 0.05 versus basal. C, alternatively, two million CFTE29o cells were transfected with 20 pmol of control or MKP-1 siRNA, incubated for 47.5 h, pretreated for 0.5 h with p38 MAPK inhibitor SB203580 or control inhibitor SB202474 (each at 0–5 μM), and stimulated for 1 h with 10 ng/ml IL-1β. After removal of IL-1β, cells were incubated for either 1.5 h (IL-8 mRNA) or 4 h (IL-8 ELISA) with SB203580 or SB202474. D–F, IL-8 production is presented as the percentage of that in cells transfected with control siRNA and preincubated with 0 μM inhibitors, p38 inh, p38 MAPK inhibitor SB203580; control inh, control inhibitor SB202474; #, p < 0.05 versus cells pretreated with 0 μM inhibitors; #, p < 0.05 MKP-1 silenced cells versus control silenced cells pretreated with 0 μM inhibitors. In A and C, the down- and upwards arrows, respectively, represent time points for intervention and obtaining of sample, and the rightwards arrows indicate the length of intervention. Data are means ± S.E.

FIGURE 7. The role of p38 MAPK in stimulated CFTE29o cells with intact or silenced MKP-1. A, CFTE29o cells were pretreated for 1 h with 10 ng/ml IL-1β. B, at 0, 5, or 60 min of stimulation, cells were collected for analysis of p38 MAPK activation by PathScan ELLISA. The p38 MAPK activation is presented as the percentage of that under basal conditions (i.e. 0 min of stimulation). *, p < 0.05 versus basal. C, alternatively, two million CFTE29o cells were transfected with 20 pmol of control or MKP-1 siRNA, incubated for 47.5 h, pretreated for 0.5 h with p38 MAPK inhibitor SB203580 or control inhibitor SB202474 (each at 0–5 μM), and stimulated for 1 h with 10 ng/ml IL-1β. After removal of IL-1β, cells were incubated for either 1.5 h (IL-8 mRNA) or 4 h (IL-8 ELISA) with SB203580 or SB202474. D–F, IL-8 production is presented as the percentage of that in cells transfected with control siRNA and preincubated with 0 μM inhibitors, p38 inh, p38 MAPK inhibitor SB203580; control inh, control inhibitor SB202474; #, p < 0.05 versus cells pretreated with 0 μM inhibitors; #, p < 0.05 MKP-1 silenced cells versus control silenced cells pretreated with 0 μM inhibitors. In A and C, the down- and upwards arrows, respectively, represent time points for intervention and obtaining of sample, and the rightwards arrows indicate the length of intervention. Data are means ± S.E.

As expected, only dexamethasone and p38 MAPK inhibitor SB603580 significantly down-regulated the cytokine-induced IL-8 (p < 0.05 versus diluent, both observations; supplemental Fig. S7B). By marked contrast, ibuprofen, JNK, or MAP2K inhibitors did not significantly alter IL-8 production (supplemental Fig. S7B). Moreover, both dexamethasone and SB603580 destabilized IL-8 mRNA to a similar degree, whereas the kinetics of mRNA decay in cells treated with ibuprofen were closer to those in diluent-treated cells (actinomycin D pulse-chase experiments; supplemental Fig. S8B).

DISCUSSION

There is a wide consensus that hyperinflammation in CF airways significantly contributes to lung damage in patients with CF (56 – 62). The exact molecular mechanisms linking CFTR mutation and hyperinflammatory responses by cells resident in CF airways are still poorly understood. Abnormal activation of transcription factors (63), the ubiquitin–protease pathway (64), 5'-adenosine monophosphate-activated protein kinase (65), and certain microRNAs (66) may be involved in hyperinflammatory responses in airway epithelial cells with mutant CFTR. Besides airway epithelial cells, macrophages expressing mutant CFTR also demonstrate abnormal inflammatory responses

4 N. Dauletbaev, D. Eklove, N. Mawji, M. Iskandar, S. Di Marco, I.-E. Gallouzi, and L. C. Lands, unpublished observations.
(67). The documented hyperinflammatory responses from both airway epithelial cells and macrophages include e.g. elevated secretion of inflammatory cytokines. Hypersecretion of inflammatory cytokines is more consistently seen in vitro under spontaneous (i.e. unstimulated) conditions (65, 67, 68) but can also present itself as an exaggerated response to pathogens (69) or a failure to terminate inflammatory responses (70). Genetic (65) or pharmacological (71) correction of mutant CFTR appears to alleviate aberrant spontaneous inflammatory responses. However, at advanced stages of CF lung disease, airway cells are exposed to both intrinsic (i.e. CFTR-related) and extraneous (i.e. bacterial, viral, and secreted inflammatory products) triggers that promote hyperinflammation. Therefore, direct pharmacological targeting of hyperinflammatory responses in CF airways is beneficial, provided these interventions are efficient and safe.

In our studies, we did not attempt to highlight the differences between inflammatory responses in CF and non-CF cells. Rather, we focused on finding common signaling cascades that are targeted by anti-inflammatory drugs to decrease production of inflammatory factors, such as IL-8.

Down-regulation of IL-8 overproduction in CF airway epithelium will limit influx of neutrophils into the airways and thus minimize the damage caused by activated neutrophils. IL-8 up-regulation by early response cytokines IL-1β and TNF-α involves several major intracellular pathways, including NF-κB and p38 MAPK. Suppression of the NF-κB pathway may not be specifically associated with down-regulation of cytokine-induced IL-8. Thus, in the previous study, we observed a comparable suppression of NF-κB by the anti-inflammatory drugs ibuprofen and dexamethasone, yet only dexamethasone down-regulates IL-8 (37). Further, other researchers have demonstrated that direct suppression of NF-κB only mildly affects IL-8 production (72). Therefore, in the present study, we focused on other pathways of IL-8 regulation as potential targets for pharmacological modulation. Specifically, we tested whether the anti-IL-8 effects of anti-inflammatory drugs are associated with inhibition of the MKP-1/p38 MAPK/mRNA stability pathway.

Both test drugs (i.e. ibuprofen and dexamethasone) were tested at clinically relevant concentrations (480 μM and 5 nm, respectively) (37). Further, the concentration of dexamethasone used by us was 200 times lower than the dose of 1 μM often utilized in experimental studies. Corticosteroids are notorious for their pleiotropic effects, and the likelihood of observing cellular phenomena irrelevant to the “true” anti-inflammatory effects of corticosteroids increases along with the dose of corticosteroids (73). Conversely, the use of low doses of dexamethasone should allow us to more easily uncover such true mechanisms. In addition, corticosteroids are often utilized clinically as a chronic basal therapy (e.g. inhaled corticosteroids) with the aim to adjust doses within the lowest possible, yet clinically effective, range. In our hands, the 5 nm concentration of dexamethasone fully retained its anti-IL-8 potential. We believe that the mechanisms exhibited by this low and effective concentration of dexamethasone may truly be the ones responsible for down-regulation of stimulated IL-8 production by inflamed airway epithelial cells.

We confirmed previous in vitro observations that normal MKP-1 levels are crucial to prevent the overproduction of IL-8 in cytokine-stimulated airway epithelial cells (53). Clinically, MKP-1 may not be diminished per se (74). However, its insufficient up-regulation by inflammatory stimuli or corticosteroids leads to similar hyperinflammatory phenotype seen by us and others (43, 49, 53) in vitro after MKP-1 silencing. Although there are no such studies in CF, several studies in patients with asthma demonstrate a diminished MKP-1 up-regulation in response to corticosteroids (74–76), more pronounced in patients with severe asthma (74). Severe asthma is associated with elevated expression of IL-8 in airway epithelium (77). It is plausible that inadequate MKP-1 up-regulation could contribute to or aggravate IL-8 overproduction.

Further, in line with previous in vitro studies (43, 49, 53), we observed that IL-8 overproduction in cells with silenced MKP-1 involves p38 MAPK. By contrast, we and others (49) ruled out the involvement of JNK in the overproduction of inflammatory factors after MKP-1 silencing. ERK, the third major class of MAPKs, is a less preferable target for MKP-1 (52) and is plausibly less relevant for the overproduction of inflammatory factors in cells with diminished MKP-1 (49). Indeed, we could not confirm the involvement of ERK in IL-8 overproduction in MKP-1 silenced cells. After we ruled out the involvement of both JNK and ERK, we focused on the role of p38 MAPK in cytokine-induced IL-8 production in cells with intact or silenced MKP-1.

Through p38 MAPK, MKP-1 can potentially control both transcriptional (i.e. through NF-κB) and post-transcriptional (i.e. through altered mRNA stability) regulation of IL-8. Others have demonstrated transcriptional mechanisms of IL-8 overproduction in MKP-1 silenced cells (43). Further supporting the transcriptional nature of IL-8 overproduction, our study ruled out increased IL-8 mRNA stability in cells with silenced MKP-1.

Hyperactivation of p38 MAPK (43) is likely the mechanism behind the decreased anti-IL-8 activity of dexamethasone in MKP-1 silenced cells observed by us. Supporting this, p38 MAPK has been implicated in resistance to corticosteroids clinically. Thus, a recent study (78) successfully used inhibitors of p38 MAPK to restore sensitivity of peripheral blood mononuclear cells to corticosteroids in patients with severe asthma.

It is clear that diminished levels of MKP-1, via p38 MAPK, lead to a hyperinflammatory phenotype. However, the efficacy of MKP-1 overexpression to curb this hyperinflammatory phenotype may not be as unequivocal. Some researchers reported a strong down-regulation of hyperinflammation by MKP-1 overexpression (43, 50). However, we and others (49) did not observe such efficient down-regulation of inflammatory responses in MKP-1 overexpressing cells. Further, the present study and work by others (42, 50) demonstrate that the magnitude of transcriptional up-regulation of MKP-1 by corticosteroids (<5-fold increase) is nowhere near the levels required for a detectable down-regulation of cytokine-induced IL-8. It is, however, possible that the corticosteroid-induced elevation of MKP-1 is well timed with respect to the inflammatory stimulus, which may be more important than the constant presence of this phosphatase at elevated levels. In addition, corticosteroids
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can further potentiate the intracellular levels of MKP-1 by increasing its half-life (41). An overexpression of MKP-1 will certainly lack this post-translational modulation feature.

Interestingly, modulation of intracellular MKP-1 levels, either by siRNA or by overexpression, did not affect IL-8 mRNA stability. Thus, the observed overproduction of IL-8 in cells with diminished MKP-1 levels is through transcriptional mechanisms.

It is intriguing that MKP-1 silencing does not potentiate mRNA stability in cytokine-stimulated cells. It is possible that IL-8 mRNA stability in cytokine-stimulated cells has reached its maximum, so that this pathway cannot be up-regulated any further.

Because MKP-1 overexpression did not affect IL-8 mRNA stability, it did not recapitulate the mechanisms of IL-8 down-regulation by dexamethasone. By contrast, direct (i.e. bypassing MKP-1) inhibition of p38 MAPK fully recapitulated the anti-IL-8 effects of dexamethasone, both in the magnitude of down-regulation and in its tentative mechanisms. Specifically, SB203580, the p38 MAPK inhibitor, demonstrated a strong anti-IL-8 potential with the IC50 comparable with dexamethasone. Next, SB203580 altered IL-8 mRNA stability in a similar manner and with a similar magnitude as dexamethasone. Importantly, neither JNK nor ERK inhibition could recapitulate these anti-inflammatory effects of dexamethasone.

We next attempted to elucidate the nature of the effects of dexamethasone on p38 MAPK. It appears that these effects do not involve diminished activation of p38 MAPK. It is possible that the anti-IL-8 effects of dexamethasone, at least in part, do not depend on diminished activation of p38 MAPK. This argument is supported by the molecular mechanisms of SB203580. In line with previous studies (79), we observed that SB203580 does not affect p38 MAPK activation (data not shown). SB203580 exerts its effects by competing with ATP binding with subsequent inhibition of p38 MAPK activity toward downstream proteins (79). This mechanism does not recapitulate the innate deactivation of the p38 MAPK pathway by MKP-1, which dephosphorylates p38 MAPK to deactivate this kinase (80).

Therefore, dexamethasone may target p38 MAPK activity or the downstream signaling cascade without affecting activation of this kinase. The mechanisms of diminished p38 MAPK activity in the presence of dexamethasone may involve the nuclear export of p38 MAPK after termination of the inflammatory stimulus, the interactions of this kinase with its downstream target proteins, or the direct effects of dexamethasone on these downstream proteins.

Of note, therapeutic measures causing a strong down-regulation of cytokine-induced IL-8 (i.e. dexamethasone and SB203580) also substantially alter IL-8 mRNA stability. By marked contrast, other measures, devoid of anti-IL-8 effects (i.e. ibuprofen and MKP-1 overexpression), are not capable of sufficiently altering mRNA stability. The significant association of anti-IL-8 effects with the capability of altering mRNA stability indicates the strong involvement of this mechanism in pharmacological down-regulation of cytokine-induced IL-8. The exact mechanisms of altered mRNA stability by dexamethasone were not examined here as these studies were beyond the hypothesis of this study.

It is worth mentioning that dexamethasone, at least at the concentration employed by us, does not prevent activation of inflammatory mechanisms by early response cytokines, such as IL-1β. Thus, the kinetics of IL-8 mRNA during stimulation were unaltered by dexamethasone. Rather, dexamethasone seems to promote cell recovery to the initial, inflammation-free status, which could be, at least in part, due to a potent destabilization of IL-8 mRNA by dexamethasone.

In conclusion, although normal levels of MKP-1 are required for a full anti-IL-8 potential of pharmacological agents, efficient pharmacological down-regulation of cytokine-induced IL-8 is linked to p38 MAPK and mRNA stability independently of MKP-1.

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