Effect of the Plant Derivative Compound A on the Production of Corticosteroid-Resistant Chemokines in Airway Smooth Muscle Cells

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Abstract

Preclinical models of human conditions including asthma showed the therapeutic potential of Compound A (CpdA), a dissociated glucocorticoid (GC) receptor (GR α) ligand. Whether CpdA inhibits GC resistance, a central feature of severe asthma, has not been addressed. We investigated whether CpdA modulates cytokine-induced GC resistance in human airway smooth muscle (ASM) cells. Healthy and asthmatic ASM cells were treated with TNF- α /IFN- γ for 24 hours in the presence or absence of CpdA. ELISA and quantitative PCR assays were used to assess the effect of CpdA on chemokine expression. Activation of GR α by CpdA was assessed by quantitative PCR, immunostaining, and receptor antagonism using RU486. An effect of CpdA on the transcription factor interferon regulatory factor 1 (IRF-1) was investigated using immunoblot, immunostaining, and small interfering RNA (siRNA) knockdown. CpdA inhibited production of fluticasone-resistant chemokines CCL5, CX3CL1, and CXCL10 at protein and mRNA levels in both asthmatic and healthy cells. CpdA failed to induce expression of GC-induced Leucine Zipper while transiently inducing mitogen-activated protein kinase phosphatase 1 (MKP-1) at both mRNA and protein levels. CpdA inhibitory action was not associated with GR α nuclear translocation,

nor was it prevented by RU486 antagonism. Activation of IRF-1 by TNF- α /IFN- γ was inhibited by CpdA. IRF-1 siRNA knockdown reduced cytokine-induced CCL5 and CX3CL1 production. siRNA MKP-1 prevented the inhibitory effect of CpdA on cytokine-induced CXCL10 production. For the first time, we show that CpdA inhibits the production of GC-resistant chemokines via GR α -independent mechanisms involving the inhibition of IRF-1 and up-regulation of MKP-1. Thus, targeting CpdA-sensitive pathways in ASM cells represents an alternative therapeutic approach to treat GC resistance in asthma.

Keywords: chemokines; transcription factors; severe asthma; asthma therapies; refractory patients

Clinical Relevance

In this study, we propose that pathways that are sensitive to the natural plant derivative Compound A represent novel targets for the treatment of corticosteroid-resistant responses in airway smooth muscle.

Approximately 5 to 10% of patients with severe asthma have difficult-to-control or severe disease that is poorly managed by glucocorticoid (GC) therapy. The management of patients with severe asthma

requires the chronic administration of high doses of GC (oral or high-dose inhaled GC), which is often associated with serious adverse effects (1). In addition, severe asthma represents a significant unmet

clinical need because these patients are responsible for more than 50% of the total asthma-related health care costs and morbidity (2–4). Therefore, better antiinflammatory therapies are urgently

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needed to properly manage GC resistance in asthma.

Despite significant findings made about the pathogenesis of severe asthma (5), the causes of GC insensitivity remain unknown. In addition to evidence showing impaired GC sensitivity in immune cells (1), the reduced response to GC therapy in severe asthma could also result from the impaired GC responses in airway structural tissues. Indeed, studies have found that steroid resistance can develop in airway epithelial cells when treated with TGF- β (6) or IL-17 (7, 8) or in airway smooth muscle (ASM) when treated with the proasthmatic cytokines TNF- α and IFN- γ (9). The production of GC-resistant mediators by ASM was also demonstrated *in vivo* by studies showing the expression of various chemokines (CX3CL1 [10], CCL11 [11], CCL15 [12], CCL19 [13]) and ADAM33 or ADAM8 (14, 15) in the ASM bundles in patients taking oral or high-dose inhaled GCs. These studies provide strong evidence for the existence of steroid-resistant pathways in ASM, which likely drive GC-resistant features in asthma. A better understanding of the mechanisms driving GC resistance in ASM could therefore lead to more effective antiasthma therapies.

Over the last decade, Compound A (CpdA), a natural compound found in the Namibian shrub, was shown to exert strong antiinflammatory actions *in vitro* and in preclinical studies via GC receptor (GR α)-dependent repression of inflammatory mediators (16–20). Importantly, these beneficial effects of CpdA were not associated with the typical side effects of GC, which affect the hypothalamic–pituitary–adrenal

(HPA) axis (19), levels of insulin (18, 19) or glucose (17), or osteoblast differentiation (21) linked to transactivation of GC-inducible genes. Recently, CpdA was found to be as effective as dexamethasone in preventing allergen responses in a murine model of asthma (22). Whether CpdA could have a therapeutic benefit in other key severe asthmatic features, such as steroid resistance, has not been investigated.

Using our established ASM model of GC insensitivity induced by TNF- α /IFN- γ (9, 23), we made the unexpected finding that CpdA suppressed the production of different fluticasone-resistant chemokines. More importantly, the therapeutic effects of CpdA occur via the activation of GR α -independent mechanisms, leading to interferon regulatory factor 1 (IRF-1) inhibition and mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) up-regulation. These studies show for the first time that CpdA-sensitive pathways are involved in driving GC resistance in ASM cells.

Materials and Methods

Culture of Human ASM Cells

ASM cells were prepared as described previously (24). Additional details regarding patient demographics are shown in Table 1.

ELISA

The concentrations of CX3CL1, CXCL10, and CCL5 in the supernatants were measured as described previously (23).

Immunofluorescence

Immunofluorescence for GR α and IRF-1 was performed as described recently (23) using anti-GR α antibody (1:200 dilution) (Santa Cruz Biotechnology, Dallas, TX), secondary antibody sheep anti-Rabbit IgG:R-Phycoerythrin (RPE) (1:10 dilution) (AbdSerotec, Kidlington, Oxfordshire, UK), anti-IRF-1 antibody (1:200 dilution) (Santa Cruz Biotechnology, Dallas, TX), and Alexa-488-labeled secondary antibody (1:300 dilution) (Invitrogen, Carlsbad, CA).

Quantitative PCR

Quantitative PCR was performed as described previously (23) and as described in the online supplement.

Small Interfering RNA Transfection

A basic Nucleofector Kit for Primary Smooth Muscle Cells (Slough, Berkshire, UK) was used for transfection studies as described previously (25) and in the online supplement.

Western Blot Analysis

Immunoblot analyses of IRF-1 or MKP-1 were performed as described previously (25, 26) using the anti-IRF-1 (C-20) and MKP-1 (C-19)-specific antibodies (Santa Cruz Biotechnology, Dallas, TX). To ensure equal loading, the membranes were stripped and reprobed with anti- β -actin antibody (C-4) (Santa Cruz Biotechnology).

Statistical Analysis

Significant differences among groups were assessed using Prism 6 with one way ANOVA, followed by *post hoc* tests (Bonferroni) or paired or unpaired *t* tests with values of $P < 0.05$ sufficient to reject the null hypothesis for all analyses.

Table 1. Demographics of Subjects Used in the Study

	Healthy	Asthma (GINA 1-5)	P Value
Number	13	23	N/A
Age, yr	36 $\pm$ 4.57	50 $\pm$ 2.01	0.0024
Sex, M/F	7/6	12/11	N/A
FEV ₁ , current	3.43 $\pm$ 0.44	2.42 $\pm$ 0.17	0.0346
FEV ₁ , % predicted	90.62 $\pm$ 6.33	76.13 $\pm$ 4.13	0.1469
FEV ₁ /FVC, %	76.5 $\pm$ 1.68	67.2 $\pm$ 2.4	0.0174
PC ₂₀ methacholine, mg/ml	13.37 $\pm$ 2.62	1.28 $\pm$ 1.1	0.0101
Atopy, <i>n</i>	3	14	N/A
ICS, mg (beclomethasone equivalent)	0	1,013.16 $\pm$ 145.28	<0.0001
OCS, <i>n</i>	0	4	N/A

Definition of abbreviations: GINA, Global Initiative for Asthma; ICS, inhaled corticosteroids; N/A, not applicable; OCS, oral corticosteroids; PC₂₀, concentration needed to produce a 20% fall in FEV₁. The bold numbers in *P* value column denote statistical significance between healthy patients and patients with asthma.

Results

CpdA Differentially Inhibits the Protein Expression of GC-Resistant Chemokines in ASM Cells

We found that the production of fluticasone-resistant CX3CL1 (Figure 1A), CXCL10 (Figure 1B), and CCL5 (Figure 1C) by TNF- α /IFN- γ was dose-dependently inhibited by CpdA (0.1–5 μ M). CpdA at 5 μ M reduced TNF- α /IFN- γ -induced CX3CL1 to 6.8 $\pm$ 0.5% and 21.97 $\pm$ 2.6% (Figure 1A), whereas CXCL10 induction was reduced to

Figure 1. Compound A (CpdA) dose-dependently suppresses production of CX3CL1 (A), CXCL10 (B), and CCL5 (C) induced by TNF- α /IFN- γ in airway smooth muscle cells from healthy subjects and subjects with asthma. Cells were pretreated with the indicated concentrations of CpdA for 2 hours before stimulation with TNF- α (10 ng/ml) and IFN- γ (25 ng/ml) for 24 hours. Chemokine levels in the supernatants of each subject performed in triplicate were assessed by ELISA. *Left panels* represent the chemokine levels in basal and TNF- α /IFN- γ -treated cells and are expressed as means $\pm$ SEM in healthy ($n = 4$; gray bars) and asthmatic cells ($n = 7$; black bars). *Right panels* represent the dose-dependent effect of CpdA expressed as percentage of TNF- α /IFN- γ -induced chemokine production. Statistical analysis was performed using one-way ANOVA (*right panels*) and Student's paired *t* test (*left panels*). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. CCL5, chemokine (C-C motif) ligand 5; CX3CL1, chemokine (C-X3-C motif) ligand 1; CXCL10, chemokine (C-X-C motif) ligand 10.

15.69 $\pm$ 1.5% and 67 $\pm$ 3.1% (Figure 1B) in healthy and asthmatic cells, respectively. Similarly, CpdA at 5 μ M reduced TNF- α /IFN- γ -induced CCL5 to basal levels in healthy and asthmatic cells, respectively (Figure 1C). It also is interesting to note that TNF- α /IFN- γ -induced chemokine production was significantly increased in ASM cells from subjects with asthma when compared with cells from healthy control subjects. Levels of CXCL10 (Figure 1B) and CCL5 (Figure 1C) were significantly increased in subjects with asthma compared with healthy control subjects (20,468 $\pm$ 681 versus 13,488 $\pm$ 204 and 3,208 $\pm$ 299 versus 1601 $\pm$ 47 pg/ml; $P < 0.05$). The inhibitory action of CpdA was not due to any cytotoxic

action because CpdA (0.1–5 μ M) failed to affect cell viability using MTT or annexin V assays (data not shown). Because no major differences in CpdA actions were seen between asthmatic (irrespective of disease severity) and nonasthmatic conditions, we presented all the subsequent studies as combined data from both asthmatic and nonasthmatic cells.

CpdA Inhibits Gene Transcription of GC-Resistant Chemokines

Quantitative PCR analyses were performed to determine whether CpdA inhibits TNF- α /IFN- γ -induced chemokine expression by acting at the transcriptional level. In cells treated with CpdA, the induction of

CX3CL1 (Figure 2A), CXCL10 (Figure 2B), and CCL5 (Figure 2C) mRNA expression by TNF- α /IFN- γ was significantly decreased to 21 $\pm$ 5.7%, 32 $\pm$ 10.2%, and 14.9 $\pm$ 2%, respectively.

CpdA Differently Regulates the Expression of GC-Inducible Genes Mitogen-Activated Protein Kinase Phosphatase-1 and Glucocorticoid-Induced Leucine Zipper

We next examined using quantitative PCR whether CpdA induced the transactivation of the GC-inducible genes MKP-1 and glucocorticoid-induced Leucine Zipper (GILZ) (27). Figure 3 shows that incubating cells with CpdA (5 μ M) for different time points (2–24 h) failed to induce the expression of the GILZ gene (Figure 3A). Fluticasone used as positive control increased GILZ expression by 22.1 $\pm$ 2.1-fold ($P < 0.001$). Interestingly, CpdA was found to induce a transient expression of the MKP-1 gene at 2 hours (5.8 $\pm$ 1.8-fold; $P < 0.05$) but to a lesser extent compared with the response seen with fluticasone (10.2 $\pm$ 1.2-fold; $P < 0.05$) (Figure 3B).

GR α Is Not Involved in CpdA Inhibitory Action

CpdA antiinflammatory effects are thought to be dependent on GR α in some cell types (16, 18, 28). Our immunostaining assays in Figure 4A show that CpdA failed to stimulate nuclear translocation of GR α when assessed at different time points (2–6 h). Fluticasone used as positive control induced a marked nuclear translocation of GR α . In addition, Figure 4B shows that GR α blockade using the glucocorticoid antagonist RU486 (1 μ M) was unable to prevent the CpdA-induced 26.4% reduction of CCL5 in response to TNF- α /IFN- γ (Figure 4C). The combination of RU486 and CpdA led to a stronger inhibitory action of CpdA (Figure 4C), an inhibitory effect that was further increased when CpdA was used at higher concentrations (>3 μ M). In contrast, RU486 completely reversed the inhibitory action of fluticasone on CCL5 expression when induced by TNF- α alone and used as a steroid-sensitive condition (29, 30).

IRF-1 Regulates TNF- α /IFN- γ -Induced CCL5 and CX3CL1 Expression

We have previously reported that IRF-1 was an important factor mediating the expression of some GC-resistant

Figure 2. CpdA blocks the transcription of chemokine gene expression induced by TNF- α /IFN- γ in airway smooth muscle cells. Cells were treated with CpdA (5 μ M) for 2 hours followed by stimulation with TNF- α (10 ng/ml) and IFN- γ (25 ng/ml) for 4 hours. Total RNA was extracted for real-time quantitative PCR. Results are expressed as percentage induction of chemokine expression by calculating the $2^{-\Delta\Delta CT}$ value. Expression of the CX3CL1 (A), CXCL10 (B), and CCL5 (C) in four donors is shown. Statistical analysis was performed using Student's paired *t* test. ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

proasthmatic proteins (25). Whether IRF-1 also regulates the expression of GC-resistant chemokines is not known. Silencing IRF-1 by $58.7 \pm 7.6\%$ using specific oligonucleotides (Figure 5A) significantly reduced TNF- α /IFN- γ -induced CCL5 mRNA expression by $60 \pm 7.3\%$, whereas mRNA levels of CX3CL1 or CXCL10 were not affected (Figure 5B). Interestingly, IRF-1 knockdown also led to a suppression of TNF- α /IFN- γ -induced CCL5 and CX3CL1 at the protein level by 45.4 ± 5.1 and 47.1 ± 14.2 , respectively (Figure 5C).

Activation of IRF-1 by TNF- α /IFN- γ Is Inhibited by CpdA

We next investigated whether an effect of CpdA on IRF-1 could explain its inhibitory

action seen against GC-resistant chemokines. We here confirm that IRF-1 was rapidly induced by TNF- α /IFN- γ , an effect seen at 1 hour and sustained up to 6 hours (Figure 6A). Immunoblot assays revealed that induction of IRF-1 by TNF- α /IFN- γ was reduced by CpdA by $34.6 \pm 9\%$ (Figure 6B). In addition, immunofluorescence staining showed that the nuclear translocation of IRF-1 in response to TNF- α /IFN- γ (Figure 6C) was also reduced by $68.3 \pm 14.7\%$ in cells treated with CpdA (Figure 6C).

CpdA Inhibits Cytokine-Induced CXCL10 Production via MKP-1

Previous studies have convincingly demonstrated the antiinflammatory action of

MKP-1 in ASM cells (26, 31). To determine the role of MKP-1 in our model of cytokine-induced GC insensitivity, we tested whether knocking down MKP-1 would affect the antiinflammatory action of CpdA. First, we confirmed by immunoblot that CpdA also increased MKP-1 at the protein level at 2 hours (Figure 7A). Interestingly, we found that knocking down MKP-1 by 52% using small interfering RNA (siRNA) (Figure 7B) completely prevented CpdA from inhibiting cytokine-induced CXCL10 production (Figure 7C) but had no effect on CCL5 (data not shown). These data suggest that MKP-1 up-regulation by CpdA has the potential to suppress the expression of the GC-insensitive chemokine CXCL10 in ASM cells.

Discussion

We have previously reported that ASM cells exposed to TNF- α /IFN- γ combination become refractory to the antiinflammatory action of corticosteroids (23, 25, 29, 32, 33). Although pathways leading to cytokine-inducible GC insensitivity are still being investigated, we here demonstrate the implication of pathways sensitive to CpdA, a natural compound initially characterized as a fully functional GR α ligand that can dissociate transactivation from transrepression (16). Interestingly, we show that the inhibitory actions of CpdA in ASM cells were not dependent on GR α and involved pathways that led to IRF-1 inhibition and up-regulation of the antiinflammatory protein MKP-1. Together these studies show for the first time that CpdA-sensitive pathways are activated by cytokines and promote steroid resistance in ASM cells.

Different preclinical studies have convincingly shown that CpdA was effective in inhibiting inflammatory conditions such as zymosan-induced inflamed paw (16), collagen-induced arthritis (18), experimental autoimmune encephalomyelitis (19, 20), and experimental autoimmune neuritis (17). These studies highlighted the lack of typical side effects associated with GC treatment and the equal efficacy of CpdA when compared with dexamethasone. The recent demonstration that CpdA was effective in a murine model of allergic asthma (22) strongly suggests that CpdA-sensitive pathways represent potential therapeutic targets for asthma. Using our ASM model of GC insensitivity, we found that CpdA

Figure 3. Differential induction of glucocorticoid response element-inducible glucocorticoid-induced Leucine Zipper (GILZ) and mitogen-activated protein kinase phosphatase 1 (MKP-1) genes by CpdA in airway smooth muscle cells. Cells were treated with CpdA (5 μ M) for 2, 4, 6, and 24 hours or fluticasone (100 nM) for 6 hours. Total RNA was extracted for real-time quantitative PCR for GILZ in three subjects (A) and MKP-1 in four subjects (B). Results are expressed as fold induction of chemokine expression by calculating the $2^{-\Delta\Delta CT}$ value for each condition. Statistical analysis was performed using one-way ANOVA. **P* < 0.05; ****P* < 0.001.

Figure 4. CpdA does not activate glucocorticoid receptor (GRα) in airway smooth muscle (ASM) cells. (A) Lack of GRα nuclear translocation by CpdA. ASM cells were treated with fluticasone (100 nM) for 2 hours and CpdA (5 μM) for 2 and 6 hours and stained for GRα or for a corresponding isotype-matched antibody. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Images are representative of data performed in cells from three different donors. (B and C) RU486 does not prevent CpdA inhibitory effect on cytokine-induced CCL5 production. (B) Cells were preincubated with RU486 (1 μM) for 10 minutes and then treated with fluticasone (100 nM) for 2 hours followed by TNF-α (10 ng/ml) for 24 hours. Ethanol 1% was used as a vehicle. Levels of CCL5 were assayed by ELISA and performed in three subjects in triplicate. **** $P < 0.0001$. (C) Cells were preincubated with RU486 (1 μM) for 10 minutes and then treated with CpdA (1 μM) for 2 hours followed by TNF-α (10 ng/ml) and IFN-γ (25 ng/ml) for 24 hours. Ethanol 1% was used as a vehicle control group. Levels of CCL5 were assayed by ELISA performed in three subjects in triplicate. Statistical analysis was performed using one-way ANOVA, with Bonferroni *post hoc* testing. ** $P < 0.01$; *** $P < 0.001$.

differentially inhibits the production of different “proasthmatic” chemokines (CCL5, CX3CL1, and CXCL10). CpdA effects were quite comparable in ASM cells from healthy and asthmatic cells irrespective of disease severity, suggesting that CpdA could be beneficial in patients with severe asthma. It is important to mention that the inhibitory profile of CpdA was different between all the tested chemokines. The magnitude of CXCL10 inhibition by CpdA was only seen at higher concentration (5 μM). In contrast, the effect of CpdA suppressive action on the other steroid-resistant CX3CL1 and CCL5 was dose dependent, was observed at much lower concentrations (~0.1–1 μM), and led

to near complete chemokine inhibition. These marked differences in CpdA inhibitory profile point to two important conclusions: (1) it suggests that different mechanisms regulate the expression of GC-insensitive chemokines, and (2) it suggests that CpdA inhibits the expression of these GC-resistant chemokines by acting on various pathways. Our observation is in agreement with previous reports showing that CpdA suppressed the expression of various proinflammatory mediators, including TNF-α (18), IL-6 (16, 21, 34), CXCL8 (21, 35), CCL2, CCL5, and CCL11 (22), with varying inhibition potencies and magnitudes. In contrast to some of these studies where dexamethasone was

equally effective as CpdA in repressing inflammatory gene expression, our study is the first to report a therapeutic action of CpdA in steroid-resistant conditions.

CpdA was originally described as a fully dissociated GRα ligand (16). We did confirm that CpdA had no transactivation potential by its failure to induce the GILZ gene (Figure 3). The transient induction by CpdA of MKP-1, another GC-inducible early gene, can be easily explained by the implication of GRα-independent mechanisms because MKP-1

up-regulation in ASM could be induced by TNF-α (36), sphingosine 1-phosphate (26), and β2 agonists (37). We found that induction of MKP-1 was also seen at the protein level. The fact that a previous report in intestinal Caco-2 cell line showed no effect of CpdA on MKP-1 expression (35) suggests that the cellular effects of CpdA are highly cell specific. Indeed, we also failed to see any GRα nuclear translocation after CpdA treatment (Figure 4), contrasting with studies (mostly from De Bosscher and collaborators) that reported GRα nuclear translocation after CpdA treatment in different cell types (17, 18, 28, 35, 38). The reasons for this discrepancy are not known, but GRα activation by CpdA appears to be highly diverse with profound differences in the kinetic of GRα nuclear translocation (seen at 30 min and at 3–6 h) and CpdA potency (concentrations from 10 nM and up to 20 μM). Increasing the incubation time with CpdA for up to 6 hours did not induce GRα nuclear translocation in ASM cells (Figure 4). Because the implication of GRα in CpdA antiinflammatory actions was also demonstrated using receptor antagonists RU38486 (39) or RU486 (28), we used the same approach but failed to see any preventive effect of RU486 on CpdA responses (Figure 4), providing strong evidence against a role of GRα in CpdA action. Our conclusion is in agreement with one previous study showing that CpdA action was still preserved in cells lacking functional GRα (GRα-deficient mouse embryonic fibroblasts or WEHI-7.1 cells transfected with siRNA GRα) (20). Although Lesovaya and colleagues showed that small hairpin RNA knockdown of GRα prevented the cytotoxic effect of CpdA in leukemia cell lines (40), the authors failed to demonstrate the presence of nuclear GRα after CpdA treatment (41).

Figure 5. IFN regulatory factor 1 (IRF-1) silencing reduces TNF- α /IFN- γ -induced expression of CCL5 and CX3CL1 but not CXCL10. Cells were transfected with Silencer Pre-designed small interfering RNA (siRNA) IRF-1 oligonucleotides or nonsilencing control scrambled siRNA (300 nM). After transfection, cells were serum deprived and stimulated with TNF- α /IFN- γ for 2 hours. (A) RNA was isolated, and levels of IRF-1 and β -actin mRNA were measured using real-time RT-PCR. Data are mean $\pm$ SEM values from three replicate samples and expressed as percentage of controls. (B) RNA from the same cells was assayed for IRF-1, CCL5, CXCL10, and CX3CL1 using real-time RT-PCR. Results are expressed as percentage mRNA remaining compared with controls (-siRNA). Data are means $\pm$ SEM from three subjects performed in triplicate. * $P < 0.05$. (C) Chemokine levels in the supernatants of scrambled and IRF-1 siRNA-transfected cells incubated with TNF- α /IFN- γ for 24 hours were assessed by ELISA. Data are means $\pm$ SEM from four donors performed in duplicate. Statistical analysis was performed using Student's paired t test. * $P < 0.05$; ** $P < 0.01$.

These observations argue against an obligatory role of GR α in CpdA effects in all cell types and support our novel observation that CpdA inhibits steroid resistance in ASM via GR α -independent mechanisms.

The observation that CpdA inhibits the expression of steroid-resistant chemokines at the mRNA level (Figure 2) strongly suggests that CpdA acted at the transcriptional level. Previous reports showed that CpdA repressed the function of different transcription factors, including NF- κ B or AP-1 (defined as transrepression) (19, 34, 40, 41), STAT6 (22), and T-bet (39). We now provide the first evidence that CpdA inhibits the

activation of IRF-1 by affecting its nuclear accumulation (Figure 6). We have previously reported that IRF-1 was key not only in driving steroid-resistant genes in ASM cells (25) but also in impairing ASM sensitivity to GC by reducing GR α transactivation function (32). Whether IRF-1 plays a role in the currently investigated GC chemokines in GC-resistant conditions has not been investigated, although limited evidence indicates a role of IRF-1 in CCL5 expression in vascular smooth muscle (42) and bronchial epithelial cells (43). Our silencing experiments confirmed the key role of IRF-1 in ASM cells in driving the expression of GC-resistant chemokines

via transcriptional (CCL5) and post-transcriptional mechanisms (CX3CL1). The post-transcriptional role of IRF-1 is interesting because a previous report performed in vascular endothelial cells showed the importance of post-transcriptional pathways in the synergistic induction of CX3CL1 by the same cytokine combination (TNF- α /IFN- γ), although the implication of IRF-1 was not investigated (44). More importantly, we made the unique finding that IRF-1 was not involved in the regulation of CXCL10 expression induced by TNF- α /IFN- γ (Figure 5). The limited evidence that IRF-1 drives CXCL10 gene expression has led to conflicting observations. Thus, previous

Figure 6. IRF-1 is time-dependently activated by TNF- α /IFN- γ and inhibited by CpDdA in ASM cells. (A) Cells were stimulated with TNF- α (10 ng/ml) and IFN- γ (25 ng/ml) for the different time points. Total cell lysates were assayed for IRF-1 or β -actin by immunoblotting assays. *Lower panel* represents means $\pm$ SEM of scanning densitometric measurement of IRF-1 expression normalized over the corresponding β -actin. Results are representative of blots performed in cells from three subjects. (B) Cells were treated with CpDdA (5 μ M) for 2 hours and then stimulated with TNF- α (10 ng/ml) and IFN- γ (25 ng/ml) for another 2 hours. Total cell lysates were assayed for IRF-1 and β -actin by immunoblot assays. The *upper panel* shows a representative IRF-1 blot, and the *lower panel* shows means $\pm$ SEM of scanning densitometric measurement of IRF-1 expression normalized over the corresponding β -actin blots performed in cells from 12 subjects (five normal subjects and seven subjects with asthma). (C) Immunofluorescence IRF-1 staining in cells stimulated with TNF- α /IFN- γ for 2 hours in the presence or absence of CpDdA added 2 hours before. The *left panel* shows a representative picture of IRF-1 staining (original magnification: $\times 20$), and the *right panel* represents the fluorescent intensity of nuclear IRF-1 staining calculated by ImageJ in 100 cells per condition. Statistical analysis was performed using one-way ANOVA (A) and Student's paired *t* test (B and C). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

reports found that IRF-1 was essential for CXCL10 expression when induced by rhinovirus in epithelial cells (45) but acted as a negative regulator of CXCL10 production in pancreatic cells when induced by cytokines (46). It is clear from these data that the role of IRF-1 in the regulation of inflammatory genes is highly complex. Nonetheless, our present report supports our initial assumption that IRF-1 is a central player in the transcription of GC-resistant genes. IRF-1 was first shown to be involved in the expression of the GC-resistant proasthmatic ectoenzyme CD38 (29). We now show that IRF-1 also regulates

expression of the GC-resistant chemokines CX3CL1 and CCL5. The lack of siRNA IRF-1 to suppress cytokine-induced CXCL10 suggests that the CpDdA inhibitory effect also involved other IRF-1-independent mechanisms. We here found that CpDdA-induced up-regulation of the antiinflammatory protein MKP-1 was crucial for the inhibition of cytokine-induced CXCL10 (Figure 7). Previous studies have confirmed the antiinflammatory potential of GC-inducible MKP-1 in human ASM cells (26, 31, 36). Our data strongly implicate MKP-1-sensitive MAPK pathways in the

regulation of CXCL10 in ASM cells. Our finding is in agreement with one previous study also performed in human healthy and asthmatic ASM cells, which showed the partial involvement of c-Jun N-terminal kinase (but not p38MAPK or extracellular signal-regulated kinase pathways) in CXCL10 production by TNF- α /IFN- γ plus IL-1 β (47). Our data also suggest that the inhibitory action of CpDdA is unlikely due to the modulation of NF- κ B or STAT-1 pathways previously shown to mediate TNF- α /IFN- γ -induced CXCL10 expression in ASM cells (48, 49). Our present report shows that multiple signaling pathways

Figure 7. MKP-1 up-regulation mediates the inhibition of cytokine-induced CXCL10 by CpdA in ASM cells. (A) Cells were stimulated with CpdA (5 μ M) for the indicated time points. Total cell lysates were assayed for MKP-1 and β -actin by immunoblot assays. (B and C) Cells were transfected with Silencer Pre-designed siRNA MKP-1 oligonucleotides or nonsilencing control scrambled siRNA (300 nM). After transfection, cells were lysed and assayed for MKP-1 and β -actin by immunoblot assays (B) or stimulated with TNF- α /IFN- γ for 24 hours in the presence or absence of CpdA (5 μ M) added 2 hours before CXCL10 levels in the supernatants were assessed by ELISA (C). Data are expressed as percentage of cytokine-induced CXCL10 expression from four donors performed in duplicate. Statistical analysis was performed using one-way ANOVA (A and C) and Student's unpaired *t* test (B). **P* < 0.05.

that are targeted by CpdA regulate the expression of GC-resistant chemokines in ASM cells. One of the main limitations of our current study is the

silencing efficiency often seen in human ASM cells, which reached ~60% for both IRF-1 and MKP-1. Therefore, we can not completely exclude a potential

role of IRF-1 in driving CXCL10 expression. Additional studies are needed to characterize the complex IRF-1 pathways that regulate expression of GC-resistant chemokines in ASM cells.

The present study demonstrates for the first time that CpdA differentially suppressed the expression of GC-insensitive chemokines in ASM cells via transcriptional and post-transcriptional mechanisms that are dependent on the antiinflammatory action of MKP-1 and inhibition of IRF-1 activity. More importantly, we show that GR α is not required for CpdA-beneficial effects as previously thought. The observation that IRF-1 is increased in the airways of patients with asthma (50), combined with studies showing that polymorphisms in IRF-1 gene are associated with increased risk of childhood asthma (51) and with IgE regulation and atopy (52), strongly indicates that IRF-1 is a potential player in the pathogenesis of asthma. Our study shows that, in addition to directly impairing GC sensitivity (25), IRF-1 also regulates the expression of a number of GC-insensitive proasthmatic genes. Understanding how CpdA suppresses IRF-1 activity could offer a novel therapeutic approach in asthma. Future studies are therefore needed to confirm whether IRF-1 is activated in the airways of patients with severe asthma and whether its expression correlates with GC insensitivity in these patients. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

- Barnes PJ. Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 2013;131:636–645.
- Durham A, Adcock IM, Tliba O. Steroid resistance in severe asthma: current mechanisms and future treatment. *Curr Pharm Des* 2011;17:674–684.
- Moore WC, Peters SP. Severe asthma: an overview. *J Allergy Clin Immunol* 2006;117:487–494, quiz 495.
- Poon AH, Eidelman DH, Martin JG, Laprise C, Hamid Q. Pathogenesis of severe asthma. *Clin Exp Allergy* 2012;42:625–637.
- Brightling CE, Gupta S, Hollins F, Sutcliffe A, Amrani Y. Immunopathogenesis of severe asthma. *Curr Pharm Des* 2011;17:667–673.
- Keenan CR, Mok JS, Harris T, Xia Y, Salem S, Stewart AG. Bronchial epithelial cells are rendered insensitive to glucocorticoid transactivation by transforming growth factor-beta1. *Respir Res* 2014;15:55.
- Zijlstra GJ, Ten Hacken NH, Hoffmann RF, van Oosterhout AJ, Heijink IH. Interleukin-17A induces glucocorticoid insensitivity in human bronchial epithelial cells. *Eur Respir J* 2012;39:439–445.
- Vazquez-Tello A, Semlali A, Chakir J, Martin JG, Leung DY, Eidelman DH, Hamid Q. Induction of glucocorticoid receptor-beta expression in epithelial cells of asthmatic airways by T-helper type 17 cytokines. *Clin Exp Allergy* 2010;40:1312–1322.
- Tliba O, Amrani Y. Airway smooth muscle cell as an inflammatory cell: lessons learned from interferon signaling pathways. *Proc Am Thorac Soc* 2008;5:106–112.
- El-Shazly A, Berger P, Girodet PO, Ousova O, Fayon M, Vernejoux JM, Marthan R, Tunon-de-Lara JM. Fractalkine produced by airway smooth muscle cells contributes to mast cell recruitment in asthma. *J Immunol* 2006;176:1860–1868.
- Ghaffar O, Hamid Q, Renzi PM, Allakhverdi Z, Molet S, Hogg JC, Shore SA, Luster AD, Lamkhioed B. Constitutive and cytokine-stimulated expression of eotaxin by human airway smooth muscle cells. *Am J Respir Crit Care Med* 1999;159:1933–1942.

12. Joubert P, Lajoie-Kadoch S, Labonte I, Gounni AS, Maghni K, Wellemans V, Chakir J, Laviolette M, Hamid Q, Lamkhioued B. CCR3 expression and function in asthmatic airway smooth muscle cells. *J Immunol* 2005;175:2702–2708.
13. Kaur D, Saunders R, Berger P, Siddiqui S, Woodman L, Wardlaw A, Bradding P, Brightling CE. Airway smooth muscle and mast cell-derived CC chemokine ligand 19 mediate airway smooth muscle migration in asthma. *Am J Respir Crit Care Med* 2006;174:1179–1188.
14. Foley SC, Mogas AK, Olivenstein R, Fiset PO, Chakir J, Bourbeau J, Ernst P, Lemiere C, Martin JG, Hamid Q. Increased expression of ADAM33 and ADAM8 with disease progression in asthma. *J Allergy Clin Immunol* 2007;119:863–871.
15. Lee JY, Park SW, Chang HK, Kim HY, Rhim T, Lee JH, Jang AS, Koh ES, Park CS. A disintegrin and metalloproteinase 33 protein in patients with asthma: relevance to airflow limitation. *Am J Respir Crit Care Med* 2006;173:729–735.
16. De Bosscher K, Vanden Berghe W, Beck IME, Van Molle W, Hennuyer N, Hapgood J, Libert C, Staels B, Louw A, Haegeman G. A fully dissociated compound of plant origin for inflammatory gene repression. *Proc Natl Acad Sci USA* 2005;102:15827–15832.
17. Zhang Z, Zhang ZY, Schluesener HJ. Compound A, a plant origin ligand of glucocorticoid receptors, increases regulatory T cells and M2 macrophages to attenuate experimental autoimmune neuritis with reduced side effects. *J Immunol* 2009;183:3081–3091.
18. Dewint P, Gossye V, De Bosscher K, Vanden Berghe W, Van Beneden K, Deforce D, Van Calenbergh S, Muller-Ladner U, Vander Cruyssen B, Verbruggen G, et al. A plant-derived ligand favoring monomeric glucocorticoid receptor conformation with impaired transactivation potential attenuates collagen-induced arthritis. *J Immunol* 2008;180:2608–2615.
19. van Loo G, Sze M, Bougarne N, Praet J, Mc Guire C, Ullrich A, Haegeman G, Prinz M, Beyaert R, De Bosscher K. Antiinflammatory properties of a plant-derived nonsteroidal, dissociated glucocorticoid receptor modulator in experimental autoimmune encephalomyelitis. *Mol Endocrinol* 2010;24:310–322.
20. Wust S, Tischner D, John M, Tuckermann JP, Menzfeld C, Hanisch UK, van den Brandt J, Luhder F, Reichardt HM. Therapeutic and adverse effects of a non-steroidal glucocorticoid receptor ligand in a mouse model of multiple sclerosis. *PLoS One* 2009;4:e8202.
21. Rauch A, Gossye V, Bracke D, Gevaert E, Jacques P, Van Beneden K, Vandooren B, Rauner M, Hofbauer LC, Haegeman G, et al. An anti-inflammatory selective glucocorticoid receptor modulator preserves osteoblast differentiation. *FASEB J* 2011;25:1323–1332.
22. Reber LL, Daubeuf F, Plantinga M, De Cauwer L, Gerlo S, Waelput W, Van Calenbergh S, Tavernier J, Haegeman G, Lambrecht BN, et al. A dissociated glucocorticoid receptor modulator reduces airway hyperresponsiveness and inflammation in a mouse model of asthma. *J Immunol* 2012;188:3478–3487.
23. Chachi L, Shikotra A, Duffy SM, Tliba O, Brightling C, Bradding P, Amrani Y. Functional KCa3.1 channels regulate steroid insensitivity in bronchial smooth muscle cells. *J Immunol* 2013;191:2624–2636.
24. Brightling CE, Ammit AJ, Kaur D, Black JL, Wardlaw AJ, Hughes JM, Bradding P. The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. *Am J Respir Crit Care Med* 2005;171:1103–1108.
25. Tliba O, Damera G, Banerjee A, Gu S, Baidouri H, Keslacy S, Amrani Y. Cytokines induce an early steroid resistance in airway smooth muscle cells: novel role of interferon regulatory factor-1. *Am J Respir Cell Mol Biol* 2008;38:463–472.
26. Che W, Manetsch M, Quante T, Rahman MM, Patel BS, Ge Q, Ammit AJ. Sphingosine 1-phosphate induces MKP-1 expression via p38 MAPK- and CREB-mediated pathways in airway smooth muscle cells. *Biochim Biophys Acta* 2012;1823:1658–1665.
27. Clark AR. Anti-inflammatory functions of glucocorticoid-induced genes. *Mol Cell Endocrinol* 2007;275:79–97.
28. Robertson S, Allie-Reid F, Vanden Berghe W, Visser K, Binder A, Africander D, Vismer M, De Bosscher K, Hapgood J, Haegeman G, et al. Abrogation of glucocorticoid receptor dimerization correlates with dissociated glucocorticoid behavior of Compound a. *J Biol Chem* 2010;285:8061–8075.
29. Tliba O, Cidlowski JA, Amrani Y. CD38 expression is insensitive to steroid action in cells treated with tumor necrosis factor-alpha and interferon-gamma by a mechanism involving the up-regulation of the glucocorticoid receptor beta isoform. *Mol Pharmacol* 2006;69:588–596.
30. Ammit AJ, Lazaar AL, Irani C, O'Neill GM, Gordon ND, Amrani Y, Penn RB, Panettieri RA Jr. Tumor necrosis factor-alpha-induced secretion of RANTES and interleukin-6 from human airway smooth muscle cells: modulation by glucocorticoids and beta-agonists. *Am J Respir Cell Mol Biol* 2002;26:465–474.
31. Quante T, Ng YC, Ramsay EE, Hennessy S, Allen JC, Parmentier J, Ge Q, Ammit AJ. Corticosteroids reduce IL-6 in ASM cells via up-regulation of MKP-1. *Am J Respir Cell Mol Biol* 2008;39:208–217.
32. Bhandare R, Damera G, Banerjee A, Flammer JR, Keslacy S, Rogatsky I, Panettieri RA, Amrani Y, Tliba O. Glucocorticoid receptor interacting protein-1 restores glucocorticoid responsiveness in steroid-resistant airway structural cells. *Am J Respir Cell Mol Biol* 2010;42:9–15.
33. Bouazza B, Krytska K, Debba-Pavard M, Amrani Y, Honkanen RE, Tran J, Tliba O. Cytokines alter glucocorticoid receptor phosphorylation in airway cells: role of phosphatases. *Am J Respir Cell Mol Biol* 2012;47:464–473.
34. De Bosscher K, Beck IM, Dejager L, Bougarne N, Gaigneaux A, Chateavieux S, Ratman D, Bracke M, Tavernier J, Vanden Berghe W, et al. Selective modulation of the glucocorticoid receptor can distinguish between transrepression of NF- κ B and AP-1. *Cell Mol Life Sci* 2014;71:143–163.
35. Reuter KC, Loitsch SM, Dignass AU, Steinhilber D, Stein J. Selective non-steroidal glucocorticoid receptor agonists attenuate inflammation but do not impair intestinal epithelial cell restitution *in vitro*. *PLoS One* 2012;7:e29756.
36. Manetsch M, Che W, Seidel P, Chen Y, Ammit AJ. MKP-1: a negative feedback effector that represses MAPK-mediated pro-inflammatory signaling pathways and cytokine secretion in human airway smooth muscle cells. *Cell Signal* 2012;24:907–913.
37. Manetsch M, Rahman MM, Patel BS, Ramsay EE, Rumzhum NN, Alkhouri H, Ge Q, Ammit AJ. Long-acting β 2-agonists increase fluticasone propionate-induced mitogen-activated protein kinase phosphatase 1 (MKP-1) in airway smooth muscle cells. *PLoS One* 2013;8:e59635.
38. Rauner M, Goettsch C, Stein N, Thiele S, Bornhaeuser M, De Bosscher K, Haegeman G, Tuckermann J, Hofbauer LC. Dissociation of osteogenic and immunological effects by the selective glucocorticoid receptor agonist, compound A, in human bone marrow stromal cells. *Endocrinology* 2011;152:103–112.
39. Liberman AC, Antunica-Noguerol M, Ferraz-de-Paula V, Palermo-Neto J, Castro CN, Druker J, Holsboer F, Perone MJ, Gerlo S, De Bosscher K, et al. Compound A, a dissociated glucocorticoid receptor modulator, inhibits T-bet (Th1) and induces GATA-3 (Th2) activity in immune cells. *PLoS One* 2012;7:e35155.
40. Lesovaya E, Yemelyanov A, Kirsanov K, Popa A, Belitsky G, Yakubovskaya M, Gordon LI, Rosen ST, Budunova I. Combination of a selective activator of the glucocorticoid receptor compound A with a proteasome inhibitor as a novel strategy for chemotherapy of hematologic malignancies. *Cell Cycle* 2013;12:133–144.
41. Lesovaya EA, Yemelyanov AY, Kirsanov KI, Yakubovskaya MG, Budunova IV. Antitumor effect of non-steroid glucocorticoid receptor ligand CpdA on leukemia cell lines CEM and K562. *Biochemistry (Mosc)* 2011;76:1242–1252.
42. Liu H, Ning H, Men H, Hou R, Fu M, Zhang H, Liu J. Regulation of CCL5 expression in smooth muscle cells following arterial injury. *PLoS One* 2012;7:e30873.
43. Matsuzaki S, Ishizuka T, Hisada T, Aoki H, Komachi M, Ichimonji I, Utsugi M, Ono A, Koga Y, Dobashi K, et al. Lysophosphatidic acid inhibits CC chemokine ligand 5/RANTES production by blocking IRF-1-mediated gene transcription in human bronchial epithelial cells. *J Immunol* 2010;185:4863–4872.
44. Matsumiya T, Ota K, Imaizumi T, Yoshida H, Kimura H, Satoh K. Characterization of synergistic induction of CX3CL1/fractalkine by TNF-alpha and IFN-gamma in vascular endothelial cells: an essential role for TNF-alpha in post-transcriptional regulation of CX3CL1. *J Immunol* 2010;184:4205–4214.

45. Hudy MH, Traves SL, Proud D. Transcriptional and epigenetic modulation of human rhinovirus-induced CXCL10 production by cigarette smoke. *Am J Respir Cell Mol Biol* 2014;50:571–582.
46. Baker MS, Chen X, Rotramel AR, Nelson JJ, Kaufman DB. Interferon regulatory factor-1 down-regulates cytokine-induced IP-10 expression in pancreatic islets. *Surgery* 2003;134:134–141.
47. Alrashdan YA, Alkhouri H, Chen E, Lalor DJ, Poniris M, Henness S, Brightling CE, Burgess JK, Armour CL, Ammit AJ, *et al.* Asthmatic airway smooth muscle CXCL10 production: mitogen-activated protein kinase JNK involvement. *Am J Physiol Lung Cell Mol Physiol* 2012;302:L1118–L1127.
48. Clarke DL, Clifford RL, Jindarat S, Proud D, Pang L, Belvisi M, Knox AJ. TNF α and IFN γ synergistically enhance transcriptional activation of CXCL10 in human airway smooth muscle cells via STAT-1, NF- κ B, and the transcriptional coactivator CREB-binding protein. *J Biol Chem* 2010;285:29101–29110.
49. Hardaker EL, Bacon AM, Carlson K, Roshak AK, Foley JJ, Schmidt DB, Buckley PT, Comegys M, Panettieri J, Reynold A., *et al.* Regulation of TNF-alpha and IFN-gamma-induced CXCL10 expression: participation of the airway smooth muscle in the pulmonary inflammatory response in chronic obstructive pulmonary disease. *FASEB J* 2003;18:191–193.
50. Sampath D, Castro M, Look D, Holtzman M. Constitutive activation of an epithelial signal transducer and activator of transcription (STAT) pathway in asthma. *J Clin Invest* 1999;103:1353–1356.
51. Nakao F, Ihara K, Kusuvara K, Sasaki Y, Kinukawa N, Takabayashi A, Nishima S, Hara T. Association of IFN-gamma and IFN regulatory factor 1 polymorphisms with childhood atopic asthma. *J Allergy Clin Immunol* 2001;107:499–504.
52. Schedel M, Pinto LA, Schaub B, Rosenstiel P, Cherkasov D, Cameron L, Klopp N, Illig T, Vogelberg C, Weiland SK, *et al.* IRF-1 gene variations influence IgE regulation and atopy. *Am J Respir Crit Care Med* 2008;177:613–621.