The plant derivative Compound A inhibits the production of corticosteroid-resistant chemokines by airway smooth muscle cells

Running title: Modulation of steroid insensitivity in the airways

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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.

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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 (GRα) 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 TNFα/IFNγ for 24 hr in the presence or absence of CpdA. ELISA and qPCR assays were used to assess the effect of CpdA on chemokine expression. Activation of GRα by CpdA was assessed by qPCR, immunostaining and receptor antagonism using RU486. An effect of CpdA on the transcription factor IRF-1 was investigated using immunoblot, immunostaining and 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 Glucocorticoid-induced Leucine Zipper (GILZ) while transiently inducing MAPK phosphatase 1 (MKP-1) at both mRNA and protein levels. CpdA inhibitory action was not associated with GRα nuclear translocation nor prevented by RU486 antagonism. Activation of IRF-1 by TNFα/IFNγ 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 GRα-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.

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Introduction

Approximately 5 to 10% of patients 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 since these patients are responsible for more than 50% of the total asthma-related health care costs and morbidity (2-4). Therefore, better anti-inflammatory therapies are urgently 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 still 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, reports have found that steroid resistance can develop in airway epithelial cells when treated with TGFβ (6) or IL-17 (7, 8) or in airway smooth muscle (ASM) when treated with "pro-asthmatic" cytokines TNFα and IFNγ (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 anti-asthma therapies.

Over the last decade, compound A (CpdA), a natural compound found in the Namibian shrub was shown to exert strong anti-inflammatory actions both *in vitro* and in preclinical studies via GC receptor (GRα)-dependent repression of inflammatory mediators

(16-20). Importantly, these beneficial effects of CpdA were not associated with GC typical side effects affecting 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 any 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 TNFα/IFNγ (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 GRα-independent mechanisms leading to IRF-1 inhibition and 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 patients' 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 GRα and IRF-1 was performed as described recently (23) using anti-GRα antibody (Santa Cruz Biotechnology; 1:200 dilution), secondary antibody Sheep anti-Rabbit IgG:RPE (AbdSerotec, 1:10 dilution), anti-IRF-1 antibody (Santa Cruz Biotechnology, dilution 1:200) and Alexa-488-labelled secondary antibody (Invitrogen, dilution 1:300).

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

Small Interfering RNA transfection: Basic Nucleofector Kit for Primary Smooth Muscle Cells 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). 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 analysis of variance ANOVA, followed by post hoc tests (Bonferonni), or paired or unpaired t-tests with values of P<0.05 sufficient to reject the null hypothesis for all analyses.

Results

CpdA differentially inhibits the protein expression of GC-resistant chemokines in ASM cells. We found that the production of fluticasone resistant CX3CL1 (**Fig. 1A**), CXCL10 (**Fig. 1B**) and CCL5 (**Fig. 1C**) by TNFα/IFNγ was dose-dependently inhibited by CpdA (0.1-5 μM). CpdA at 5 μM reduced TNFα/IFNγ-induced CX3CL1 to 6.8 ± 0.5 and 21.97 ± 2.6 % (**Fig. 1A)** while CXCL10 induction was reduced to 15.69 ± 1.5 and 67 ± 3.1% (**Fig. 1B**) in healthy and asthmatic cells respectively. Similarly, CpdA at 5 μM reduced TNFα/IFNγ-induced CCL5 to basal levels in healthy and asthmatic cells respectively (**Fig. 1C**). It also is interesting to note that TNFα/IFNγ-induced chemokine production was significantly increased in ASM cells from asthmatics when compared to cells from healthy controls. Levels of CXCL10 (**Fig. 1B**) and CCL5 (**Fig. 1C**) were significantly increased in asthma compared to healthy controls (20468 ± 681 vs 13488 ± 204 and 3208 ± 299 vs 1601 ± 47 pg/ml, P<0.05). The inhibitory action of CpdA was not due to any cytotoxic action as CpdA (0.1-5 μM) 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 non-asthmatic conditions, we presented all the subsequent studies as combined data from both asthmatic and non-asthmatic cells.

CpdA inhibits gene transcription of GC-resistant chemokines. Quantitative PCR analyses were performed to determine whether CpdA inhibits TNFα/IFNγ-induced chemokine expression by acting at the transcriptional level. In cells treated with CpdA, the induction of CX3CL1 (**Fig. 2A**), CXCL10 (**Fig. 2B**) and CCL5 (**Fig. 2C**) mRNA expression by TNFα/IFNγ was significantly decreased to 21 ± 5.7, 32 ± 10.2 and 14.9 ± 2 %, respectively.

CpdA differently regulates the expression of GC-inducible genes mitogen-activated protein kinase phosphatase-1 (MKP-1) and Glucocorticoid-induced leucine zipper (GILZ). We next examined using qPCR whether CpdA induced the transactivation of GC-inducible genes MKP-1 and GILZ (27). **Figure 3** shows that incubating cells with CpdA (5 μM) for different time points (2-24 hr) failed to induce the expression of GILZ gene (**Fig. 3A**). Fluticasone used as positive control increased GILZ expression by 22.1 ± 2.1 fold (P<0.001). Interestingly, CpdA was found to induce a transient expression of MKP-1 gene at 2 hr (5.8 ± 1.8 fold, P<0.05) but to a lesser extend compared to the response seen with fluticasone (10.2 ± 1.2 fold, P<0.05) (**Fig. 3B**).

GRα is not involved in CpdA inhibitory action. CpdA anti-inflammatory effects are thought to be dependent on the GC receptor (GRα) in some cell types (16, 18, 28). Our immunostaining assays in **Figure 4A** show that CpdA failed to stimulate nuclear translocation of GRα when assessed at different time points (2-6 hr). Fluticasone used as positive control induced a marked nuclear translocation of GRα. In addition, **Figure 4B** shows that GRα blockade using the glucocorticoid antagonist RU486 (1 μM) was unable to prevent the CpdA-induced 26.4% reduction of CCL5 in response to TNFα/IFNγ (**Fig. 4C**). Combination of RU486 and CpdA led to a stronger inhibitory action of CpdA (**Fig. 4C**), an inhibitory effect that was further increased when CpdA was used at higher concentrations (>3 μM). In contrast, RU486 completely reversed the inhibitory action of fluticasone on CCL5 expression when induced by TNFα alone used as a steroid-sensitive condition (29, 30).

The transcription factor IRF-1 regulates TNFα/IFNγ-induced CCL5 and CX3CL1 expression. We have previously reported that IRF-1 was an important factor mediating the expression of some GC resistant pro-asthmatic proteins (25). Whether IRF-1 also regulates the expression of GC resistant chemokines is not known. Silencing IRF-1 by

58.7 ± 7.6% using specific oligonucleotides (**Fig. 5A**) significantly reduced TNFα/IFNγ induced CCL5 mRNA expression by 60 ± 7.3 % while mRNA levels of CX3CL1 or CXCL10 were not affected (**Fig. 5B**). Interestingly, IRF-1 knockdown also led to a suppression of TNFα/IFNγ induced CCL5 and CX3CL1 at the protein level by 45.4 ± 5.1 and 47.1 ± 14.2, respectively (**Fig. 5C**).

Activation of IRF-1 by TNFα/IFNγ 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 TNFα/IFNγ, an effect seen at 1 hr and sustained up to 6 hr (**Fig. 6A**). Immunoblot assays revealed that induction of IRF-1 by TNFα/IFNγ was reduced by CpdA by 34.6 ± 9 % (**Fig. 6B**). In addition, immunofluorescence staining showed that the nuclear translocation of IRF-1 in response to TNFα/IFNγ (**Fig. 6C**) was also reduced by 68.3 ± 14.7 % in cells treated with CpdA (**Fig. 6C**).

CpdA inhibits cytokine-induced CXCL10 production via MKP-1. Previous studies have convincingly demonstrated the anti-inflammatory 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 anti-inflammatory action of CpdA. First, we confirmed by immunoblot that CpdA also increased MPK-1 at the protein level at 2hr (**Fig. 7A**). Interestingly, we found that knocking down MKP-1 by 52% using siRNA (**Fig. 7B**) completely prevented the capacity of CpdA to inhibit cytokine-induced CXCL10 production (**Fig. 7C**) but had no effect on CCL5 (data not shown). These data suggest that MKP-1 upregulation 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 TNFα/IFNγ combination become refractory to the anti-inflammatory 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 GRα ligand that can dissociate transactivation from transrepression (16). Interestingly, we show that the inhibitory actions of CpdA in ASM cells were not dependent on GRα and involved pathways that led to IRF-1 inhibition and up-regulation of the anti-inflammatory 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 to 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 differentially inhibits the production of different "pro-asthmatic" 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 severe patients. 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, 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. First, it suggests that different mechanisms regulate the expression of GC-insensitive chemokines. Second, 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 pro-inflammatory mediators including TNFα (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 GILZ gene (Fig. 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 as MKP-1 induction in ASM could be induced by TNFα (36), sphingosine 1-phosphate (26), and β2-agonists (37). We found that induction of MPK-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 following CpdA treatment (Fig. 4) contrasting with studies (mostly from De Bosscher and collaborators) who reported GRα nuclear translocation following CpdA treatment in different cell types (17, 18, 28, 35, 38). The reasons for this discrepancy is 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, 3 hr to 6 hr) and CpdA potency (concentrations from 10 nM and up to 20 μM). Increasing the incubation time with CpdA for up to 6 hr did not induce any GRα nuclear translocation in ASM cells (Fig. 4). Because the implication of GRα in CpdA anti-inflammatory actions was also demonstrated using receptor antagonists RU38486 (39) or RU486 (28), we also used the same approach but failed to see any preventive effect of RU486 on CpdA responses (Fig. 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 et al., showed that shRNA knockdown of GRα prevented the cytotoxic effect of CpdA in leukaemia cell lines (40) the authors failed to demonstrate the presence of nuclear GRα following CpdA treatment (41). These observations argue against an obligatory role of GRα in CpdA effects in all cell types and support our novel observation that CpdA inhibits steroid resistance in ASM via GRα independent mechanisms.

The observation that CpdA inhibits the expression of steroid resistant chemokines at the mRNA levels (Fig. 2) strongly suggests that CpdA acted as the transcriptional level. Previous reports showed that CpdA repressed the function of different transcription factors including NF-κB 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 (Fig. 6). We have previously reported that IRF-1 was key not only in driving steroid resistant genes in ASM cells (25) but also impairing ASM sensitivity to GC by reducing GRα 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 reported a role of IRF-1 in CCL5 expression in both 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 as a previous report performed in vascular endothelial cells did show the importance of post-transcriptional pathways in the synergistic induction of CX3CL1 by the same cytokine combination (TNFα/IFNγ), 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 TNFα/IFNγ (Fig. 5). The limited evidence showing implication of IRF-1 in driving CXCL10 gene expression has led to conflicting observations. Thus, previous 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 implication 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 pro-asthmatic ectoenzyme CD38 (29). We now show that IRF-1 also regulates expression of GC-resistant chemokines CX3CL1 and CCL5 (present report). The lack of siRNA IRF-1 to suppress cytokine-induced CXCL10 suggests that CpdA inhibitory effect also involved other IRF-1-independent mechanisms. We here found that CpdA-induced up-regulation of the anti-inflammatory protein MKP-1 was crucial for the inhibition of cytokine-induced CXCL10 (**Fig. 7**). Previous elegant studies have indeed confirmed the anti-inflammatory potential of GC-inducible MKP-1 in human ASM cells (26, 31, 36). Our data strongly indicates the implication of 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 JNK (but not p38MAPK or ERK pathways) in CXCL10 production by TNFα/IFNγ plus IL-1β (47). Our data also suggest that the inhibitory action of CpdA is unlikely due to the modulation of NF-κB or STAT-1 pathways previously shown to mediate TNFα/IFNγ-induced CXCL10 expression in ASM cells (48, 49). Our present report shows that multiple signalling pathways 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. As such, we can also not completely exclude a potential role of IRF-1 in driving CXCL10 expression. Additional studies are clearly 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 posttranscriptional mechanisms that are dependent on the anti-inflammatory action of MKP-1 and inhibition of IRF-1 activity. More importantly, we show that GRα is not required for CpdA beneficial effects as previously thought. The observation that IRF-1 is increased in the airways of asthmatic patients (50) combined to 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 pro-asthmatic 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 severe asthmatics and its expression correlates with GC insensitivity in these patients.

Funding

Author contributions

	Healthy	Asthma (GINA 1-5)	p value
Number	13	23	N/A
Age	36 ± 4.57	50 ± 2.01	**0.0024**
Gender (M/F)	7/6	12/11	N/A
FEV1 current	3.43 ± 0.44	2.42 ± 0.17	**0.0346**
FEV1 (% predicted)	90.62 ± 6.33	76.13 ± 4.13	0.1469
FEV/FVC (%)	76.5 ± 1.68	67.2 ± 2.4	**0.0174**
PC_{20} methacholine (mg/ml)	13.37 ± 2.62	1.28 ± 1.1	**0.0101**
Atopy (no)	3	14	N/A
ICS mg (beclomethasone equivalent)	0	1013.16 ± 145.28	< 0.0001
OCS (no)	0	4	N/A

Table 1: Demographics of subjects used in the study. ICS, inhaled corticosteroids, OCS, oral corticosteroids**,**

Figure legends

Figure 1. CpdA dose-dependently suppresses production of CX3CL1 (A), CXCL10 (B) and CCL5 (C) induced by TNFα/IFNγ in ASM cells from healthy and asthmatic subjects. Cells were pre-treated with the indicated concentrations of CpdA for 2 hr prior stimulation with TNFα (10 ng/ml) and IFNγ (25 ng/ml) for 24 hr. Chemokine levels in the supernatants of each subject performed in triplicate were assessed by ELISA assays. Left panels represent the chemokine levels in basal and TNFα/IFNγ treated cells and expressed as means ± SEM in healthy (n=4, grey bars) and asthmatic cells (n=7, black bars). Right panels represent the dose-dependent effect of CpdA expressed as % of TNFα/IFNγ–induced chemokine production. Statistical analysis was performed using one-way ANOVA (right panels) and student paired t-test (left panels). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 2. CpdA blocks the transcription of chemokine gene expression induced by TNFα/IFNγ in ASM cells. Cells were treated with CpdA (5μM) for 2 hr, followed by stimulation with TNFα (10 ng/ml) and IFNγ (25 ng/ml) for 4 hr. Total RNA was extracted for real time quantitative PCR. Results are expressed as % induction of chemokine expression by calculating the negative inverse of the $2^{-\Delta\Delta CT}$ value. Expression of the CX3CL1 **(A)**, CXCL10 **(B)** and CCL5 **(C)** in n= 4 donors. Statistical analysis was performed using student paired t-test. ****P < 0.0001.

Figure 3. Differential induction of GRE-inducible GILZ and MKP-1 genes by CpdA in ASM cells. Cells were treated with CpdA (5 μM) for 2, 4, 6 and 24 hr or fluticasone (100 nM) for 6 hr. Total RNA was extracted for real time quantitative PCR for GILZ in n=3 subjects (**A**) and MKP-1 in n=4 subjects (**B**). Results are expressed as fold induction of chemokine expression by calculating the negative inverse of the $2^{-\Delta\Delta CT}$ value for each

condition. Statistical analysis was performed using one-way ANOVA. ***P < 0.001, *P<0.05.

Figure 4. CpdA does not activate GRα in ASM cells. A) Lack of GRα nuclear translocation by CpdA. ASM Cells were treated with Fluticasone (100 nM) for 2 hr and CpdA (5 µM) for 2 and 6 hr, stained for GRα or corresponding isotype matched antibody. Nuclei were stained with DAPI. Images are representative of data performed in cells from n = 3 different donors. **B-C)** RU486 does not prevent CpdA inhibitory effect on cytokine-induced CCL5 production. **B)** Cells were pre-incubated with RU486 (1 µM) for 10 minutes then treated with fluticasone (100 nM) for 2 hr followed by TNFα (10 ng/ml) for 24 hr. Ethanol 1% was used as a vehicle. Levels of CCL5 were assayed by ELISA performed in n = 3 subjects in triplicate. ****P<0.0001, ***P<0.001, **P<0.01. **C)** Cells were pre-incubated with RU486 (1µM) for 10 minutes then treated with CpdA (1 µM) for 2 hr followed by TNFα (10 ng/ml) and IFNγ (25 ng/ml) for 24 hr. Ethanol 1% was used as a vehicle control group. Levels of CCL5 were assayed by ELISA performed in n = 3 donors in triplicate. Statistical analysis was performed using one-way ANOVA, with Bonferroni post hoc testing. *P<0.05, **P < 0.01.

Figure 5. IRF-1 silencing reduces TNFα/IFNγ-induced expression of CCL5 and CX3CL1 but not CXCL10. Cells were transfected with Silencer Pre-designed siRNA IRF-1 oligonucleotides or non-silencing control scrambled siRNA (300 nM). Following transfection, cells were serum deprived and stimulated with TNFα/IFNγ for 2 hr. **A)** RNA was then isolated and levels of IRF-1 and β-actin mRNA were measured using real-time RT-PCR. Data are mean ± SEM values from n=3 replicate 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 % mRNA remaining compared to controls

(-siRNA). Data are mean ± SEM values from n=3 subjects performed in triplicate. *P<0.05

C) Chemokine levels in the supernatants of scrambled and IRF-1 siRNA transfected cells incubated with TNFα/IFNγ for 24 hr were assessed by ELISA assays. Data are mean ± SEM values from n=4 donors performed in duplicate. Statistical analysis was performed using student paired t-test. *P<0.05, **P<0.01.

Figure 6. IRF-1 is time-dependently activated by TNFα/IFNγ and inhibited by CpdA in ASM cells. A) Cells were stimulated with TNFα (10 ng/ml) and IFNγ (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 ± SEM of scanning densitometric measurement of IRF-1 expression normalized over the corresponding β-actin. Results are representative of blots performed in cells from 3 subjects. **B)** Cells were treated with CpdA (5 µM) for 2 hr, then stimulated with TNFα (10 ng/ml) and IFNγ (25 ng/ml) for another 2 hr. Total cell lysates were assayed for IRF-1 and β-actin by immunoblot assays. Upper panel shows a representative IRF-1 blot and lower panel shows means ± SEM of scanning densitometric measurement of IRF-1 expression normalized over the corresponding β-actin blots performed in cells from 12 subjects (5 normal and 7 asthmatics). **C)** Immunofluorescence IRF-1 staining in cells stimulated with TNFα/IFNγ for 2 hr in the presence or absence of CpdA added 2 hr before. Left panel shows a representative picture of IRF-1 staining (20X magnification) and 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 paired t-test (B, C). **P < 0.01.

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 µM) for the indicated time points. Total cell lysates were assayed for MKP-1 and β-actin by immunoblot assays. **B-C)**

Cells were transfected with Silencer Pre-designed siRNA MKP-1 oligonucleotides or non-silencing control scrambled siRNA (300 nM). Following transfection, cells were lysed and assayed for MKP-1 and β-actin by immunoblot assays **(B)** or stimulated with TNFα/IFNγ for 24 hr in the presence or absence of CpdA (5 µM) added 2 hr before CXCL10 levels in the supernatants were assessed by ELISA assays **(C)**. Data are expressed as % of cytokine-induced CXCL10 expression from n=4 donors performed in duplicate. Statistical analysis was performed using one-way ANOVA (A, C) and student unpaired t test (B). $*P<0.05$

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