

Protein phosphatase 5 mediates corticosteroid insensitivity in airway smooth muscle in patients with severe asthma

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Abstract

Background: The mechanisms driving glucocorticoids (GC) insensitivity in patients with severe asthma are still unknown. Recent evidence suggests the existence of GC insensitive pathways in airway smooth muscle (ASM) caused by a defect in GC receptor (GR α) function. We examined whether other mechanisms could potentially explain the reduced sensitivity of ASM cells to GC in severe asthmatics.

Methods: ASM cells from healthy and severe asthmatic subjects were treated with TNF α and responses to corticosteroids in both cohorts were compared by ELISA, immunoblot, immunohistochemistry and real time PCR. Immunohistochemistry and flow cytometry assays were used to assess the expression of the protein phosphatase PP5 in endobronchial biopsies and ASM cells.

Results: The production of CCL11 and CCL5 by TNF α was insensitive to both fluticasone and dexamethasone in ASM cells from severe asthmatic compared to that in healthy subjects. Fluticasone-induced GR α nuclear translocation, phosphorylation at serine 211 and expression of Glucocorticoid-induced leucine zipper (GILZ) was significantly reduced in ASM cells from severe asthmatics compared to responses in healthy subjects. Levels of PP5 were increased in ASM cells from severe asthmatics and PP5 knockdown using siRNA restored fluticasone repressive action on chemokine production and its ability to induce GR α nuclear translocation and GRE-dependent GILZ expression. In vivo PP5 expression was also increased in the ASM bundles in endobronchial biopsies in severe asthmatics.

Conclusions: PP5-dependent impairment of GR α function represents a novel mechanism driving GC insensitivity in ASM in severe asthma.

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Key words: protein phosphatase, corticosteroid insensitivity, airway smooth muscle, corticosteroid receptor, receptor phosphorylation.

Introduction

It is estimated that approximately 5 to 10% of asthmatic patients have difficult-to-control or severe disease that is poorly managed by current anti-asthma drugs including glucocorticoid (GC) therapy. Despite a significant progress made about the pathogenesis of severe asthma, including the identification of distinct phenotypes (1), GC insensitivity remains the main therapeutic challenge and little is known about the mechanisms affecting GC therapy in these patients. Evidence for the existence of signaling defects potentially responsible for the loss of GC efficacy in severe asthma was first reported in studies assessing GC function in immune cells, including alveolar macrophages, T cells, and peripheral blood mononuclear cells (PBMC) (2, 3). These studies reported not only a reduction in GC responsiveness in cells from severe asthmatic patients but also the potential mechanisms including a defective nuclear translocation of GC receptor ($GR\alpha$) (4). This abnormal $GR\alpha$ signaling was later linked to an increased p38MAPK-dependent receptor phosphorylation on key residues such as serine 226 (5, 6). Other studies also reported a profound reduction in HDAC activity (7) as well as an increased expression of $GR\beta$ (8) as additional mechanisms that explain the loss of GC sensitivity in patients with severe asthma. Altogether, these studies support the interesting concept that the reduced sensitivity to GC therapy seen in severe asthma could result from an abnormal GC signaling in key inflammatory cells. Whether GC insensitivity in severe asthma also results from the reduced anti-inflammatory actions of GCs in non-immune cells such as airway smooth muscle (ASM) is an important question worth exploring considering the clinical benefit provided by removing ASM in severe asthma using bronchial thermoplasty (9).

The question of an impaired GC response in airway structural tissues, and more specifically in ASM, the main effector tissue regulating the bronchomotor tone (10), has been also raised. Whether this GC insensitive state exists in ASM *in vivo* in patients with severe

asthma was indirectly supported by reports showing the expression of various inflammatory mediators by the ASM bundles in patients treated with high doses of inhaled and/or oral GCs (11-14). We have shown in different studies that $\text{TNF}\alpha/\text{IFN}\gamma$ can impair ASM cell sensitivity GCs by blocking their ability to suppress various mediators including chemokines (15-19). We also identified that the protein phosphatase PP5, known to negatively regulate $\text{GR}\alpha$ function in some cell types (20), was a key player in blunting GC sensitivity in healthy ASM cells (16, 17). These studies provided the initial evidence for the development of GC “insensitive state” in healthy ASM cells and a possible role of the inflammatory cytokines. More recent studies convincingly showed that this GC insensitive phenotype was also present “constitutively” in cultured ASM cells isolated from severe asthmatics. Indeed, two reports demonstrated a decreased efficacy of dexamethasone to suppress either the production of “pro-asthmatic” chemokines (21) or the proliferative capacity (22) in ASM cells from severe asthma when compared to cells from non-severe asthma. Although the underlying mechanisms have yet to be determined, the same group recently reported that the reduced sensitivity to GC seen in ASM cells of severe asthma was in part due to an abnormal GC signaling as evidenced by the impaired nuclear translocation of $\text{GR}\alpha$ (23).

In the present study, we provide additional evidence to support the existence of abnormal GC sensitivity *in vitro* in ASM cells derived from severe asthmatics. In contrast to the initial reports, we found a completely different mechanism of GC insensitivity in ASM cells of severe patients. We demonstrate that fluticasone failed to induce $\text{GR}\alpha$ phosphorylation in severe asthma due to a high constitutive level of serine threonine phosphatase PP5. The clinical relevance of these findings was shown by the increased expression of PP5 seen in the ASM bundles in the endobronchial biopsies of severe asthmatics. These data show for the first time that an abnormal PP5 axis plays a central role in blunting GC responsiveness in ASM cells of severe asthmatics and suggests that PP5-

mediated GC insensitivity in ASM cells could drive the pathogenesis of severe asthma via the production of GC-insensitive inflammatory chemokines.

Materials and Methods

Subjects' characteristics

Severe asthmatic subject and healthy volunteers were recruited. Asthmatic subjects had a consistent history and objective evidence of asthma as indicated by one or more of the following: (1) a positive methacholine challenge test defined as a concentration of nebulized methacholine causing a 20% drop in FEV1 of <8 mg/mL, (2) diurnal maximum peak flow variability of >20% over 2 week time, and (3) improvement of >15% in FEV1 15 minutes after bronchodilator therapy (24). Asthma severity was defined according to British Guideline on the Management of Asthma treatment steps (24). Subjects receiving step 4 (high dose inhaled corticosteroids, long-acting bronchodilators) and 5 (as per step 4 with oral corticosteroids) treatment were included in this study. The studies were approved by the Leicestershire, Northamptonshire, and Rutland Research Ethics Committee (references: 4977, 04/Q2502/74 and 08/H0406/189). Immunohistochemistry study used a cohort of severe asthma patients post oral GC treatment (2 weeks of prednisolone, study number UHL-09789). Written informed consent was gained from all participants prior to their involvement. Tables 1 and 2 show the demographics of the patients used in the immunohistochemistry studies and molecular/cellular studies, respectively.

Fiberoptic bronchoscopy

Subjects underwent fiberoptic bronchoscopy, as described previously (17). All biopsy samples were from the GSK-sponsored studies RES100767 (clinicaltrials.gov: NCT00331058) and RES100769 (clinicaltrials.gov: NCT00327197).

Culture of human ASM cells

Primary human ASM cells were obtained from healthy subjects and severe asthma patients and isolated from endobronchial biopsy specimens from severe asthma patients, as previously

described (25). All studies were performed in cultured ASM cells in their 4th-6th passages and serum starved for 24 hr prior the experiments.

ELISA

ELISA was performed as described previously (17) on 50 µl cell supernatants using the R&D Systems DuoSet kits according to the manufacturer's instructions.

Western blot

Immunoblot was performed as described previously (17) using antibodies against phospho-GR α -serine 211 (Cell Signalling), GR α (BD Bioscience). To ensure equal loading, all membranes were stripped and reprobed with anti- β -actin (Santa Cruz Biotechnology). Band intensities visualized by the ECL system were quantified using Image J software.

Quantitative PCR

Quantitative PCR was performed as described previously (17) using the following primers:

β -actin forward: 5'-CCCAAGGCCAACCGCGAGAAGAT-3' and reverse: 5'-GTCCCGGCCAGCCAGGTCCAG-3'; glucocorticoid-induced leucine zipper (GILZ), forward: 5'-TCTGCTTGGAGGGGATGTGG-3' and reverse: 5'-ACTTGTGGGGATTCGGGAGC-3'. PP5 primers (considered as proprietary information, cat# sc-44602-PR, 540 bp) were purchased from Santa Cruz Biotech.

Flow Cytometry

Confluent ASM cells were lifted with Acutase, then were fixed and permeabilized in 4% paraformaldehyde/0.1% saponin for 15 min on ice. Cells were then stained with either 2 µg/ml mouse anti-human PP5 Ab (BD Bioscience, UK) or isotype-matched control (Mouse IgG; DAKO, UK) overnight, followed by secondary anti-Mouse Alexa Fluor® 488 conjugate

(Life Technology, UK) for 1h. Staining was examined by flow cytometry using the Becton Dickinson FACScan (Oxford, U.K.).

Small Interfering RNA transfection

Transfection studies were performed using Amaxa Nucleofector II device (program D-033; Amaxa Biosystems, Germany) as described in (16). $1.5\text{--}2 \times 10^6$ ASM cells were transfected with small interfering RNA (siRNA) to PP5 (100 nM, Santa Cruz Biotech.) and non-silencing scrambled siRNA (100 nM, Santa Cruz Biotech.).

Immunohistochemistry

Mucosal biopsy specimens were processed into glycol methacrylate (Polysciences, Northampton, UK). Three-micrometer sections were stained with antibody against human PP5 (mouse monoclonal, $2\mu\text{g/ml}$, BD Bioscience), α -smooth muscle alpha actin (Millipore UK) or isotype control (Dako, UK). The EnVision FLEX kit (Dako, UK) was used for the staining of the sections. The quantitative assessment of PP5 staining in the ASM bundle was performed using a thresholding technique based on the 3,3'-Diaminobenzidine (DAB) staining that was evaluated using the Image J software as described in our previous study (26).

Statistical analysis

Statistical analysis was performed with PRISM software, version 6 (GraphPad Software, La Jolla, Calif). The Wilcoxon matched pairs test was used for intragroup analysis before and after cytokine treatment. Parametric data (effects of dexamethasone and fluticasone on cytokine production) were analysed using one-way or two way analysis of variance (ANOVA) with the Dunnett's multiple comparison, or when appropriate the unpaired t test. Nonparametric data were analyzed with the Kruskal-Wallis test and the Dunn test for post hoc comparison. A P value of less than 0.05 was considered significant.

Results

Impaired inhibition of TNF α -induced CCL11 and CCL5 by dexamethasone and fluticasone in ASM cells from severe asthmatics. **A-C-E,** Production of two chemokines was significantly elevated in ASM cells treated with TNF α (10 ng/ml, 24 hr). In ASM cells from control subjects (n=5), levels of CCL11 (**Fig. 1A**), CCL5 (**Fig.1C**) and CXCL10 (**Fig.1E**) were increased from 238 \pm 189, 152 \pm 37 and 966 \pm 300 pg/ml to 3492 \pm 710, 367 \pm 89, and 13050 \pm 1872 pg/ml, respectively. In ASM cells from severe asthmatics (n=7), levels of CCL11 (**Fig.1A**), CCL5 (**Fig.1C**) and CXCL10 (**Fig.1F**) were increased from 395 \pm 230, 90.9 \pm 15 and 1675 \pm 736 pg/ml to 5961 \pm 2023, 310 \pm 55 pg/ml and 18435 \pm 6050 pg/ml, respectively. No significant difference was noticed in the basal and induced chemokine levels between control subjects and severe asthmatics. **B-D-F,** In cells from healthy controls (n=5), induction of CCL11 by TNF α was dose-dependently inhibited by both dexamethasone (LogEC50=-8.32 \pm 0.86) and fluticasone (LogEC50=-9.12 \pm 0.92), reaching a maximum suppression of 78.6% and 81.2% at 10⁻⁶M corticosteroid, respectively (**Fig.1B**). Similarly, induction of CCL5 (**Fig.1D**) by TNF α was also sensitive to dexamethasone (LogEC50=-9.11 \pm 0.30) and fluticasone (LogEC50=-8.94 \pm 0.45) with a maximum chemokine suppression of 92.6% and 87% at 10⁻⁶M corticosteroid, respectively. In contrast, induction of CCL11 and CCL5 by TNF α was totally insensitive to dexamethasone or fluticasone in ASM cells from severe asthmatics (n= 7, **Fig.1B** and **1D**). No differences were seen between severe asthma and healthy controls in the repressive effect of dexamethasone on cytokine-induced CXCL10 production (**Fig.1F**) (LogEC50=-8.942 \pm 0.7450 versus -8.629 \pm 0.4612).

Ligand-induced GR α nuclear translocation, GR α phosphorylation and GRE-dependent gene expression is impaired in ASM cells from severe asthmatics. We next assessed whether GR α function was impaired in ASM cells from severe asthmatics. We tested whether ligand-induced GR α nuclear translocation was affected in severe asthma as recently

suggested by others (23). Our immunostaining results in **Figure 2A** show that in contrast to ASM cells from healthy donors where fluticasone induced GR α nuclear translocation in 100% of cells, this response was reduced by 77% of cells from severe asthmatics ($P<0.0001$). **Fig.2B** shows that the ability of fluticasone to time-dependently induce GR α phosphorylation at serine 211 in ASM cells was also dramatically reduced in severe asthmatics ($n=3$) when compared to control subjects ($n=3$). Ligand-induced GR α phosphorylation in ASM cells from patients with severe asthma was also reduced by 70%, 82.4%, 81.25% and 81.5% at 2, 4, 6 and 24 hr post-stimulation respectively when compared to responses in cells from healthy subjects ($P<0.05-0.001$) (**Fig.2B**). To further explore the possibility that GR α transcriptional activity was altered in severe asthmatics, we assessed whether expression of glucocorticoid-induced Leucine Zipper (GILZ) (**Fig.2C**), a well-defined GRE-inducible gene (17), was affected in severe asthma. We found that induction of GILZ seen at 2, 4, 6 and 24 hr post-fluticasone was significantly reduced at all-time points in ASM cells from severe asthmatics when compared to cells from healthy controls ($P<0.01$).

PP5 expression is increased in ASM cells from severe asthmatics and mediated GC insensitivity. We have previously shown that the increased expression of PP5 was a key factor driving cytokine-induced GC insensitivity in healthy ASM cells (16, 17). We now show using both quantitative PCR and flow cytometry analyses that PP5 expression was significantly increased at the transcriptional level (**Fig.3A**, $P<0.001$) as well as at the protein level (**Fig.3B**, $P<0.01$) in ASM cells from severe asthmatics when compared to cells from healthy controls. In addition, we used siRNA to investigate the role of PP5 in the reduced GC sensitivity in ASM cells from severe asthmatics using the same knockdown approach previously reported by us (16). We found that that PP5 was knockdown by more than 85% using siRNA (**Fig.4A**, $P<0.01$) and by more than 65% at the protein level when assessed by flow cytometry. Interestingly, compared to cells transfected with scrambled siRNA controls,

cells transfected with PP5 siRNA had enhanced GC responses as evidenced by the 25% augmented GILZ expression (**Fig.4A**, middle panel) and 9 fold increase over basal in GR α nuclear translocation (**Fig.4A**, right panel). There was also a restoration of fluticasone's ability to inhibit TNF α -induced production of CCL11 (**Fig.4B**, P<0.05) and CCL5 (**Fig.4C**, P<0.01).

Increased PP5 expression in ASM bundles in severe asthma. We next used immunohistochemistry applied to endobronchial biopsies to determine whether PP5 expression in ASM bundles was increased *in vivo* in severe asthmatics as reported *in vitro* in ASM cells (**Fig.3**). A marked staining of PP5 was observed in ASM bundles when compared to the staining in isotype control sections (**Fig. 5A**). The overall intensity of PP5 staining was significantly increased in ASM bundles from severe asthmatics (n=20) compared to that in healthy controls (n=8) (**Fig. 5C**, P=0.0056). In addition, no difference in the proportion of ASM bundles (stained with α -smooth muscle alpha actin and related to the total biopsy area) was detected between severe asthmatics and healthy controls (**Fig. 5B**).

Discussion

Two recent reports convincingly demonstrated that GC insensitivity was present in ASM cells from severe asthmatics and this loss of GC function was mostly due to an impaired nuclear translocation of GR α (21, 23). Although ASM cells from our cohort of severe asthmatics had a reduced sensitivity to GC in terms of CCL5 and CCL11 inhibition, we found that this GC insensitivity was due to an impairment of GR α phosphorylation and transactivation properties. Not only were levels of the serine/threonine protein phosphatase PP5 elevated in cells from severe asthmatics but inhibiting PP5 using siRNA restored ASM cell sensitivity to GCs. In addition, PP5 expression was found to be increased in ASM bundles in endobronchial biopsies of severe asthmatics who had been taking oral GC therapy for 2 consecutive weeks. Together, the present study provides the first evidence for a role of steroid-insensitive PP5 pathways in the development of GC insensitivity in ASM cells from subjects with severe asthma.

The evidence that systemic GCs exert a direct modulatory action on ASM *in vivo* that could participate in their therapeutic actions in asthma could be drawn from the conclusions of a recent double-blind intervention study performed in patients with stable asthma. The authors found that following a 14-days therapy with oral prednisolone (0.5mg/kg/day) there was a striking change in 15 genes in ASM, 2 of which (called FAM129A and SYNPO2) were associated with an improvement of airway hyper-responsiveness (27). One can therefore speculate that the persistent expression of different pro-inflammatory/pro-remodelling factors such as IL-33 (28), ADAM33 (12), CCL19 (11), TSLP (14) and Pentraxin 3 (13) seen *in vivo* in ASM tissues of severe asthmatics despite oral GC therapy may be explained by the defective anti-inflammatory actions of GCs in ASM in these patients. We now found that the expression of PP5, a protein phosphatase previously shown by some groups to antagonize

GR α function (20), was highly expressed *in vivo* in ASM from patients with severe asthma despite a course of oral prednisolone (Fig. 5). Thus, in addition to producing various inflammatory/pro-remodelling mediators, ASM from patients with severe asthma also expressed *in vivo* other GC insensitive proteins that are capable of potentially altering ASM sensitivity to GC therapy. The role of protein phosphatases in asthma has been investigated mostly in animal models or immune cells such as alveolar macrophages or peripheral blood mononuclear cells and has focussed on another member of the protein phosphatase family called PP2A. These studies identified PP2A deficiency as an important player in driving GC insensitive features such as airway hyper-responsiveness and cytokine production (29, 30). Despite controversies on whether PP5 exerts a positive or negative influence on GR α activity (20), some convincing reports found that aberrant expression of PP5 can blunt GC responsiveness in various cell types including breast cancer cells (31) and mouse embryonic fibroblasts (32) and in A459 cells (33). More recently, we have provided strong evidence in human ASM cells (from healthy subjects) that increasing expression of PP5 (but not that of PP2A) contributed to the impaired GC responsiveness seen in our experimental model of GC insensitivity (e.g., cells exposed to TNF α /IFN γ) (16, 17). We here demonstrate there is an abnormal PP5 expression in ASM cells of severe asthmatics that is responsible for their reduced sensitivity to GCs.

We found that while dexamethasone and fluticasone dose-dependently inhibited production of CCL11 and CCL5 by ASM cells in healthy donors (EC₅₀~0.1-1nM range), their anti-inflammatory action (even at high concentrations) was surprisingly lost in patients with severe asthmatic patients (Fig. 1). Our study further supports the existence of a defective GC sensitivity in ASM cells in patients with severe asthmatics recently reported by two studies. The authors showed a reduced efficacy of dexamethasone to suppress chemokine production (CCL11 and CXCL8) induced by TNF α (21) or cell proliferation induced by FCS/TGF β (22).

The mechanism initially proposed to explain GC refractoriness seen in cells from severe asthmatics was an impaired ligand-induced GR α nuclear translocation while no changes in receptor phosphorylation was noticed (23). This defective GR α nuclear translocation was associated with a failure of dexamethasone to inhibit TNF α -induced p65 recruitment to CCL11 promoter and reduced baseline GR α levels (23). In contrast to this study, we failed to see any difference in baseline GR α levels in cells from severe asthmatics (data not shown). As shown by Chang and colleagues (23), we also found that fluticasone-induced GR α nuclear translocation was significantly impaired by more than 70% in severe asthma. More importantly, the ability of fluticasone to induce i) GR α phosphorylation at serine 211 residues and ii) GR α -dependent transactivation, measured by fold induction of GC inducible GILZ gene, was also impaired in cells from severe asthmatics. This latter observation is consistent with the large body of evidence showing that ligand-induced GR α phosphorylation at serine 211 residues is essential for the maximal transactivation of the GR α (34, 35). This concept of impaired ligand-induced GR α transactivation as a mechanism for GC insensitivity was recently observed in isolated monocytes from steroid-resistant asthmatics (36). The authors found that induction of MKP-1, another GC inducible gene, was also blunted in steroid-resistant asthmatics compared to steroid sensitive patients. Together, we propose that GC insensitivity seen in ASM cells from severe asthmatics is caused by two main mechanisms leading to impaired GR α transactivation that include i) a decreased receptor phosphorylation (present study) and ii) a reduced receptor nuclear translocation (present study and (23)).

Studies using our inducible model of GC insensitivity consisting of healthy ASM cells treated with TNF α /IFN γ allowed us to demonstrate that GR α transactivation and phosphorylation at serine 211 residues were markedly decreased because of an increased expression of PP5 (15-17). This observation prompted us to investigate whether PP5 was

indeed playing any role in the reduced GC responsiveness seen in ASM cells from severe asthmatics. Interestingly, we made the surprising finding that PP5 was significantly greater in ASM cells from severe asthmatics that displayed a reduced sensitivity to GCs. The fact that mRNA levels of PP5 were also increased by ~7 fold in cells from severe asthmatics strongly suggests that transcriptional mechanisms are behind this elevated PP5 expression. Because PP5 mediated GC insensitivity in healthy ASM cells induced by $\text{TNF}\alpha$ and $\text{IFN}\gamma$ (16, 17), two cytokines known to be associated with severe asthma (37-39), it is quite possible that the increased PP5 expression seen in severe asthma is a consequence of a direct action of different inflammatory cytokines on ASM bundles. More importantly, we found that ASM cells responsiveness to fluticasone (in terms of CCL11 and CCL5 suppression) could be fully restored following PP5 downregulation using siRNA strategy (Fig.4). The finding that GC-induced $\text{GR}\alpha$ nuclear translocation and GRE-dependent GILZ expression was increased by PP5 siRNA (Fig.4A) further confirms a key role played by PP5 in the modulation of GC sensitivity by acting at different levels on $\text{GR}\alpha$ signalling pathways. The regulation of $\text{GR}\alpha$ nuclear translocation by PP5 could derive from its ability to interact with the motor protein dynein associated with microtubules shown to be essential for $\text{GR}\alpha$ movement by some groups (20). This results combined to the observation of an increased *in vivo* expression of PP5 in ASM bundles of severe asthmatics argues for a novel role of PP5 axis in the development of GC insensitivity seen in ASM cells in severe asthma.

One limitation of the present study is the use of mostly male subjects in the immunohistochemistry studies which only became apparent after the results were obtained since the entire procedure was undertaken using a blinded approach (Table 2). Due to the limitation of available biopsies it was impossible to increase the number of female subjects. However, based on the observation that impaired *in vitro* GC sensitivity in ASM cells was equally seen in both male and female subjects with severe asthma and PP5 expression was

also increased in the biopsies of 2 female asthma patients, it suggests that PP5 could also be abnormal in female patients with severe asthma. Nonetheless, future studies will be needed to determine whether the increased PP5 expression observed in ASM from severe asthma can also be reproduced in larger number of female patients. Because severe asthma is a condition usually treated with oral GC, it could be argued that the dampened ASM cell responsiveness to GCs seen in our severe asthmatics was due to prior treatment with GC which could have influenced our results by causing a down-regulation of GR α (40). First, while GC insensitivity was seen in cells from all seven severe asthmatics, only 3 patients were treated with oral GC. Second, the expression of another chemokine CXCL10 was found to be equally sensitive to GC in severe asthmatics versus healthy controls (**Fig.1E and 1F**). The reasons why CXCL10 production could still be repressed by fluticasone indicates that some of the anti-inflammatory mechanisms of GC can be preserved in cells from severe asthmatics. Although GR α signalling was strongly impaired in the severe asthmatics, it was not completely abrogated thus potentially explaining their remaining anti-inflammatory action. This lack of complete inhibition of GC signalling could also explain the same paradoxical observation reported for another chemokine called CX3CL1 which was significantly enhanced by GC in TNF α /IFN γ -treated ASM cells (41, 42) and in ASM cells from severe asthmatics (21) despite GC-induced GR α phosphorylation and nuclear translocation being significantly blunted (17, 23). In addition, we and others showed the implication of distinct pathways that regulate CXCL10 in ASM cells. Thus, in contrast to CCL5, CCL11 and CX3CL1, the production of CXCL10 in GC resistant conditions was the only chemokine which was i) not repressed by a combination of fluticasone and KCa3.1 channel blockers (17), ii) poorly sensitive to the natural derivative compound A (43) and iii) not involving IRF-1-dependent pathways (43). This differential action of GCs on CXCL10 expression was also reported in epithelial cells where fluticasone increased cytokine-induced CXCL10 while

inhibiting production of CXCL8 (44). These observations combined to the emerging fact that modulation of GR α activity by PP5 is highly gene-specific (20) potentially explain why despite aberrant PP5 pathways in severe asthma GC regulation on CXCL10 expression is unaffected. These observations clearly demonstrate that the expression of chemokine genes in cells from severe asthma is differentially regulated by GCs due to the implication of PP5-sensitive and insensitive mechanisms.

We therefore suggest that, in addition to a defective GR α nuclear translocation previously suggested by others (23), GC insensitivity found in ASM cells in severe asthma also involves a PP5-dependent suppression of GR α phosphorylation and transcriptional activities. Our study demonstrates for the first time that targeting PP5 axis may offer a new therapeutic option of the treatment of severe asthmatics who are refractory to GC therapy (1).

Figure legends

Figure 1: Impaired suppression of TNF α -induced CCL11 and CCL5 by fluticasone and dexamethasone in ASM cells from patients with severe asthma. **A-C**, Growth-arrested ASM cells were treated with 10ng/ml TNF α for 24 hr. Levels of CCL11 (**A**), and CCL5 (**C**), CXCL10 (**E**) in the supernatants (each assessed in triplicate) of n=5 healthy controls and n=7 severe asthmatics were examined by ELISA as described in *Methods*. Horizontal lines represent medians.*P<0.05 and **P<0.01, versus basal levels. **B-F**, Growth-arrested ASM cells were pre-treated with the indicated concentrations of dexamethasone or fluticasone for 2 hr then stimulated with 10 ng/ml TNF α for 24 hr. Levels of CCL11 (**B**), CCL5 (**D**) and CXCL10 (**F**) in the supernatants (each assessed in triplicate) were examined by ELISA as described in *Methods*. Data are expressed as the means \pm SEM in ASM cells from n=7 severe asthma and n=5 healthy controls, all performed in triplicate.*P<0.05 and **P<0.01 versus healthy controls.

Figure 2: Fluticasone-induced GR α nuclear translocation, GR α phosphorylation and GRE-dependent gene expression is impaired in ASM cells from severe asthma. **A**, Growth-arrested ASM cells were treated with fluticasone (100 nM) for 6 hr and stained for GR α . Nuclei were stained with DAPI. The *left panels* show representative images of GR α nuclear translocation in one healthy subject and one severe asthmatic. The *right panel* shows the percentage (%) of nuclei stained with GR α post-fluticasone treatment counted in 100 cells per field in n = 3 healthy subjects and n = 4 severe asthmatics. ***P<0.001 versus healthy controls. **B**, Growth-arrested ASM cells ASM cells from healthy controls and patients with severe asthma were treated with 100 nM fluticasone for the indicated time points, total cell lysates were prepared and assayed for total GR α , phospho-GR α at serine 211 and β -actin as loading control by immunoblot analysis. Representative immunoblot (upper panel) and the

corresponding scanning densitometry analyses of blots (lower panel) of phospho-GR α -Ser211 from n=3 healthy subjects and n=3 patients with severe asthma, with each value normalized over the mean density of the corresponding β -actin. The results are expressed as means \pm SEM. *P<0.05, **P<0.01 and ***P<0.001 versus healthy controls. **C**, Total mRNA (2 μ g) from growth-arrested ASM cells ASM cells treated with fluticasone for the indicated time points was subjected to real-time PCR with GILZ and β -actin primers. Each experiment was performed in duplicate and repeated in cells from n= 3 different healthy donors and n=5 patients with severe asthma. The results are expressed as means \pm SEM. **P<0.01 versus healthy controls.

Figure 3: Increased expression of PP5 in ASM cells from subjects with severe asthma. A, Total RNA was extracted from growth-arrested ASM cells in n = 5 healthy controls and n = 5 patients with severe asthma and levels of PP5 and GAPDH were measured by real-time PCR. Results are expressed as means \pm SEM. ***P<0.001 versus healthy controls. **B**, Representative histogram showing PP5 expression assessed by flow cytometry in one healthy control and one patient with severe asthma. **B**, Quantitative analysis of PP5 levels assessed in n=5 healthy controls and n=5 subjects with severe asthma. Data are expressed as means \pm SEM, **P<0.01 versus healthy controls.

Figure 4: Reducing expression of PP5 in patients with severe asthma enhanced GC-induced GILZ expression, GR α nuclear translocation and restores fluticasone inhibition of CCL11 and CCL5. A, Growth-arrested ASM cells from n = 3 severe asthmatics were transfected with Silencer predesigned siRNA PP5 or non-silencing control scrambled siRNA (100 nM). After transfection, cells were lysed and assayed for PP5 by real time PCR. Results are expressed as means \pm SEM. ***P<0.001 versus cells transfected with scrambled siRNA. Growth arrested ASM cells from severe asthmatics transfected with scrambled or PP5 siRNA (100 nM) were treated with 100 nM fluticasone for 4 hr. Cells were used for **C**, assessing

GILZ expression by real-time PCR. Each experiment was performed in duplicate and repeated in cells from $n = 4$ patients with severe asthma. The results are expressed as means \pm SEM. * $P < 0.05$ versus scrambled siRNA controls; **D**, GR α nuclear translocation was determined by assessing the staining intensity of GR α in the nucleus of cells from $n = 4$ patients with severe asthma. The results are expressed as means \pm SEM. * $P < 0.05$ versus Fp stimulated cells transfected with scrambled siRNA; ** $P < 0.01$ versus basal cells transfected with PP5 siRNA. **B-C**, CCL11 (**B**) and CCL5 (**C**) in the supernatants of transfected cells with either PP5 siRNA or scramble siRNA and stimulated with TNF α (10 ng/ml, 24 hr) in the presence or the absence of fluticasone (100 nM) were determined by ELISA. Results are shown as means \pm SEM of experiments performed in triplicate in $n = 3$ patients with severe asthma. * $P < 0.05$, ** $P < 0.01$ versus respective control siRNA.

Figure 5: Increased *in vivo* expression of PP5 in ASM bundles in patients with severe asthma. Representative immunostaining in bronchial biopsies for PP5, α -smooth muscle actin or isotype control antibody (data not shown) in one healthy control (**A**, left panels) or one patient with severe asthma (**A**, right panels) at magnification X200. Positive staining was visualised with DAB detection (with hematoxylin counter stain) and quantified using the thresholding technique with Image J software. **B**, % ASM area per biopsy in healthy controls ($n=8$) and patients with severe asthma ($n=20$). *NS*, no statistical difference. **C**, PP5 staining within ASM bundle expressed as % staining per total area of ASM in $n = 8$ healthy controls and $n = 20$ patients with severe asthma. Data represented as means \pm SEM, P value versus healthy controls.

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