ERJ Express. Published on August 4, 2011 as doi: 10.1183/09031936.00017911

IL-17A induces glucocorticoid insensitivity in human bronchial epithelial cells

G. Jan Zijlstra, BSc[1]; Nick H.T. ten Hacken, MD, PhD[2]; Roland F. Hoffmann, MSc[1]; Antoon J.M. van Oosterhout, PhD[1]*; Irene H. Heijink, PhD[1;2]*

[1]Laboratory of Allergology and Pulmonary Diseases, Department of Pathology and Medical Biology [2]Department of Pulmonology, GRIAC research institute, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands.

*Share senior authorship

Running head: IL-17A-induced glucocorticoid insensitivity

Corresponding author:

Prof. Antoon J.M. van Oosterhout, PhD

Laboratory of Allergology and Pulmonary Diseases

Department of Pathology and Medical Biology

GRIAC research institute, University Medical Center Groningen, University of Groningen

PO Box 30001, 9700 RB Groningen, The Netherlands

Tel: +31(0)50-3610335

Fax: +31(0)50-3619007

e-mail: a.j.m.van.oosterhout@med.umcg.nl

Abbreviations

GC, Glucocorticoid; GR, Glucocorticoid Receptor; GRE, Glucocorticoid Response Element; HDAC, Histone Deacetylase; IL, Interleukin; PI3K, phosphoinositide-3-kinase

Abstract

A subset of asthma patients suffers from glucocorticoid (GC)-insensitivity. Th17 cells have an emerging role in GC-insensitivity, although the mechanisms are still poorly understood.

We investigated whether IL-17A induces GC-insensitivity in airway epithelium by studying its effects on responsiveness of TNF-α-induced IL-8 production to budesonide in human bronchial epithelial 16HBE cells. We unravelled the underlying mechanism by the use of specific pathway inhibitors, reporter- and overexpression constructs and an HDAC activity assay.

We demonstrate that IL-17A-induced IL-8 production is normally sensitive to GCs, while IL-17A pre-treatment significantly reduces the sensitivity of TNF-α-induced IL-8 production to budesonide. IL-17A activated the p38, ERK and phosphoinositide-3-kinase (PI3K) pathways, and the latter appeared to be involved in IL-17A-induced GC-insensitivity. Furthermore, IL-17A reduced HDAC activity, and overexpression of HDAC2 reversed IL-17A-induced GC-insensitivity. In contrast, IL-17A did not affect budesonide-induced transcriptional activity of the GC receptor, suggesting that IL-17A does not impair the actions of the ligated GC receptor.

In conclusion, we show for the first time that IL-17A induces GC-insensitivity in airway epithelium, which is likely mediated by PI3K activation and subsequent reduction of HDAC2 activity. Thus, blockade of IL-17A or downstream signalling molecule PI3K may offer new strategies for therapeutic intervention in GC-insensitive asthma.

Introduction

Asthma is an obstructive lung disorder, characterized by airway hyperreactivity, airway remodelling and invasion of inflammatory cells, e.g. eosinophils, mast cells, and T helper cells. Inhaled glucocorticoids (GCs) are currently the most effective anti-inflammatory treatment for asthma. However, a subset of asthmatic subjects is relatively insensitive to this treatment (1;2).

GCs exert a broad spectrum of anti-inflammatory effects upon binding to their receptor (GR). The ligated receptor translocates to the nucleus and suppresses pro-inflammatory gene transcription by recruitment of histone deacetylases (HDACs). HDAC2 in particular is able to induce deacetylation of histones containing inflammatory genes, thereby restricting access of the transcriptional machinery to these genes and inhibiting transcription (3). In addition to the recruitment of HDACs, GCs are able to exert anti-inflammatory effects through activation of GC response elements (GREs), which are present in the promoter of several anti-inflammatory genes, inducing their transcription.

Reduced sensitivity to GCs has been clinically associated with neutrophilic airway inflammation (4;5), but it is still largely unclear which cellular and molecular mechanisms contribute to this GC-insensitivity. Th17 lymphocytes have an emerging role in the induction of neutrophilic airway inflammation (6). Moreover, Th17-induced neutrophilic airway inflammation in mice was reported to be GC-insensitive (7). Th17 cells act by producing various inflammatory cytokines, including IL-17A, IL-17F, IL-21, and IL-22, which act locally to induce pro-inflammatory transcription in tissue cells, e.g. IL-8 (6). Interestingly, IL-17A is known to enhance chemotaxis of neutrophils by inducing IL-8 release in bronchial epithelial cells (8) and airway smooth muscle cells (9). Despite these novel insights, it is still unknown why GCs are

unable to efficiently suppress Th17-mediated inflammation. Although neutrophils are considered relatively unresponsive to GCs, the production of chemokines involved in neutrophilic infiltration, e.g. IL-8 by airway epithelium, can be suppressed by GCs (10).

Therefore, we hypothesize that Th17-mediated GC-insensitive inflammation is not mediated by direct induction of GC-insensitive neutrophil chemoattractant production, but by IL-17A inducing epigenetic changes diminishing the possibilities for GCs to inhibit cytokine production. In the present study, we demonstrate that IL-17A significantly reduces GC-responsiveness in the bronchial epithelial cell line 16HBE, which is likely mediated by activation of PI3K signalling and subsequent reduction in HDAC2 activity.

Methods

Cell culture

16HBE14o- human bronchial epithelial cells, (16HBE; kindly provided by dr. D.C. Gruenert, University of California, San Francisco) were cultured on flasks coated with 30 μg/ml collagen and 10 μg/ml BSA in Eagle's Minimal Essential Medium (EMEM; Lonza, Walkersville, MD, USA) containing 10% fetal calf serum (FCS; Hyclone, Logan, UT) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Biowhittaker, Verviers, Belgium) as previously described (11).

Stimulation of the cells

Cells were seeded in duplicates at a concentration of 10^5 cells/mL in 24-well plates, grown to ~90% confluence and serum-deprived overnight. For measurement of IL-8 levels, cells were pre-treated for 2 hours with budesonide (BUD, AstraZeneca, Lund, Sweden) in concentrations ranging from 10^{-11} to 10^{-9} M, washed, and subsequently stimulated with/without 10 ng/mL IL-17A (R&D Systems, Abingdon, UK) or 10 ng/mL TNF-α (Sigma, St. Louis, MO) for 24 hours. Alternatively, IL-17A was added 2 hours prior to BUD treatment, in presence and absence of specific inhibitors of ERK (U0126, 10 μM), p38 (SB203580, 1 μM) and PI3K (LY294002, 10 μM) pathways, which was added 30 min prior to IL-17A incubation. All inhibitors were purchased from Tocris Bioscience (Bristol, UK). For measurements of GRE-mediated transcription, cells were incubated for 2h with 10 ng/ml IL-17A and subsequently incubated with BUD in a concentration ranging from 10^{-9} M to 10^{-7} M.

Measurement of IL-8 production

Cell-free supernatants were harvested 24 hour after stimulation with IL-17A or TNF-α. IL-8 production was measured by ELISA (R&D Systems) according to manufacturer's instructions.

Immunodetection

Upon stimulation with/without IL-17A (10 ng/ml) for 5-120 min, total cell lysates were obtained by resuspension of cells in sample buffer (2% SDS, 10% glycerol, 2% 2-mercapto-ethanol, 60 mM Tris-HCl (pH 6.8) and bromophenol blue) and boiling for 5 minutes. Proteins were separated by SDS-PAGE and blotted on a nitrocellulose membrane. Immunodetection was performed with anti-phospho-p38, anti-phospho-ERK, anti-phospho-Akt (Cell Signaling Technology, Hitchin, Herts, UK) and pan-ERK (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (11).

HDAC activity

Cells were stimulated for 2 hours with 10 ng/ml IL-17A, resuspended in radio-immunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS and 50 mM Tris pH 8.0, and sonicated to obtain total cell lysates. Protein levels were determined using BCA kit (Thermo Scientific, Rockford, IL, USA). HDAC activity was assessed by a Fluorometric HDAC activity assay (BioVision, Mountainview, CA, USA) performed according to manufacturer's instructions.

Transfection with GRE luciferase construct

The GRE-Luciferase construct was kindly provided by Dr. S.A. Asgeirsdóttir (University Medical Center Groningen, Groningen, The Netherlands). The thymidine kinase-driven Renilla luciferase vector (pRL-TK, Clonetech, Paris, France) was used as internal control. 16HBE cells were grown to 80-90% confluence in EMEM containing 10% FCS. For GRE promoter activity measurements, cells were transfected with OptiMEM (Lonza), 1 μL/mL lipofectamine, 1000 ng/mL of GRE-Luciferase and 100 ng/mL of Renilla luciferase construct. After 20 hours, cells were washed, followed by stimulation with indicated amounts of budesonide for 24h. Next, cells were lysed in 50 μl Passive Lysis Buffer (Promega, Madison, WI, USA) and subjected to freezing/thawing. Firefly and Renilla luciferase activities were determined using the Dual-Luciferase Reporter Assay Kit (Promega) on a Luminoskan Ascent microplate luminometer (Thermo Scientific) according to manufacturer's instructions. Results were normalized by dividing the Firefly luciferase activity with the Renilla luciferase activity of the same sample.

Transfection with HDAC2 construct

Cells were grown to 80-90% confluence in EMEM containing 10% FCS, transfected overnight in OptiMEM with 1 μL/mL lipofectamine and 1000 ng/mL of an pcDNA3.1-HDAC2 construct (kindly provided by Dr. K. Ito, Imperial College, London, UK) or an empty vector. After transfection, stimulations were performed as described above.

Statistical analysis

- We tested for normal distribution with the Shapiro-Wilk normality test and observed that TNF-α-induced IL-8 production by 16HBE cells was normally distributed. We used the Student's t-test for paired observations to test for statistical significance and the ANOVA with Dunnett's post test when we compared different concentrations.

Results

IL-17A-induced IL-8 production in 16HBE cells is normally sensitive to budesonide

First, we tested whether IL-17A induces IL-8 production in 16HBE cells. We observed that treatment with increasing doses of IL-17A (24 hours) induces an approximately 2-fold increase in IL-8 production (Fig. 1a). To explore the possibility that IL-17A induces IL-8 production through a GC-insensitive pathway, we tested whether IL-17A-induced IL-8 production is sensitive to budesonide. We observed that budesonide dose-dependently decreases IL-17A-induced IL-8 production by 16HBE cells, resulting in a reduction of 49.6% ±7.2% at a concentration of 10^{-9} M (Fig. 1b). 10^{-9} M budesonide reduced TNF-α-induced IL-8 production to a similar extent, e.g. by 46.7% ± 7.1 (Fig. 2), indicating that IL-17A-induced IL-8 production in bronchial epithelium is normally sensitive to GCs.

IL-17A induces GC-insensitivity of TNF-α-induced IL-8 production

Next, we aimed to determine whether IL-17A is able to induce GC-insensitivity of TNF-α-induced IL-8 production in 16HBE cells and we assessed the effect of pre-treatment with 10 ng/mL IL-17A prior to the addition of budesonide. 2h pre-treatment with IL-17A did not affect baseline or TNF-α-induced IL-8 levels (Fig. 2), but importantly, it significantly reduced the inhibitory effect of $3*10^{-10}$ M budesonide, from 34.1% ± 6.7 to 12.5 % ± 5.8 ($p=0.006$, $n=4$, Fig. 2). Furthermore, we observed that pre-treatment with IL-17 increased the IC50 of budesonide on TNF-α-induced IL-8 production from $5.20*10^{-10}$ to $1.15*10^{-9}$ M (*n=4,* $p<0.05$, data not shown). This indicates that IL-17A pre-treatment reduces sensitivity of TNF-α-induced IL-8 production to budesonide. Increasing the period of incubation (up to 6 hours) or

altering the concentration of IL-17A (1-100 ng/ml) did not further affect GC-sensitivity (data not shown).

IL-17A induces phosphorylation of ERK, p38 and Akt

To unravel the molecular mechanisms involved in the reduced responsiveness of IL-8 production to budesonide upon IL-17A treatment, we first studied whether IL-17A is able to activate PI3K/Akt, MEK/ERK-1/2 and p38 MAPK signalling in 16HBE cells. Immunodetection revealed that IL-17A induces phosphorylation of Akt, ERK-1/2 and p38 in 16HBE cells, with a peak between 5 to 20 min (Fig. 3a). Analysis by densitometry revealed that levels of p-Akt, p-ERK and p-p38 were significantly increased upon 5 min of incubation with IL-17 (Fig. 3b-d).

Inhibition of PI3K abrogates IL-17A-induced GC-insensitivity

Next, we aimed to determine whether activation of these pathways is involved in IL-17A-induced GC-insensitivity. We used the specific inhibitors LY294002, U0126, and SB203580 to inhibit the PI3K, MEK/ERK, and p38 pathways, respectively. Notably, IL-17A-induced GC-insensitivity was, at least partially, reversed upon addition of the PI3K inhibitor LY294002, resulting in stronger inhibition of IL-8 production by budesonide in presence of IL-17A (from 1.8% ± 9.0 to 24.9% ± 6.6, p=0.04). In contrast, the effect of IL-17A on GC-sensitivity was not significantly affected by the presence of U1026 and SB203580 (Fig. 3~~b~~e). These data indicate that PI3K, but not ERK and p38, activity contributes to the effect of IL-17A on GC-sensitivity.

Reduced HDAC activity may be involved in IL-17A-induced GC-insensitivity

Since PI3K has been implicated in the phosphorylation of HDAC2, leading to its inactivation and a subsequent reduction in GC-sensitivity (12), we were interested to see whether IL-17A can reduce HDAC activity in bronchial epithelium. We analyzed HDAC activity upon 2 hours of incubation with or without IL-17A. Importantly, IL-17A significantly reduced HDAC activity compared to untreated cells (Fig. 4a). In contrast to HDAC activity, IL-17 did not significantly affect total protein expression of HDAC2 (data not shown).

Since HDAC2 has previously been described as the predominant HDAC involved in GC action, we were interested to further underscore the role of HDAC2 in IL-17A-induced GC-insensitivity and we studied the effect of IL-17A on GC-insensitivity upon overexpression of HDAC2. In cells expressing the control vector, budesonide ($3*10^{-10}$ M) inhibited TNF-α-induced IL-8 production by 14.2% ± 4.3. Pre-treatment with IL-17A significantly reduced this effect to 0.6% ± 5.4. In contrast, IL-17A was not able to reduce the effect of budesonide in cells overexpressing HDAC2, as budesonide still reduced TNF-α-induced IL-8 production by 11.1% ± 3.2 (Fig. 4b).Overexpression of HDAC1 and HDAC3 did not result in a significant abrogation of the IL-17-mediated effect (see supplementary figure 1). These data further support the role for reduced HDAC2 activity in IL-17A-induced GC-insensitivity.

IL-17A has no effect on GRE-mediated transcription

Another mechanism that has been implicated in cytokine-induced GC-insensitivity is the reduced translocation of the ligated GR (13). Nuclear translocation of the ligated GR is required for GCs to exert their suppressive effect on transcription pro-

inflammatory genes as well as on transcription of anti-inflammatory genes that contain a GRE in their promoter (3;13). To test whether IL-17A affects transcriptional activity of the GR, we determined the effect of IL-17A treatment on a GRE reporter construct in 16HBE cells. Our data demonstrate that budesonide induces a dose-dependent increase in GRE transcriptional activity, which reached an approximately 7-fold increase at $3*10^{-8}$ M budesonide over basal GRE transactivation ($p<0.001$). Pre-treatment with IL-17A did not affect budesonide-induced activity of the GRE promoter at any of the used concentrations (Fig. 5). This indicates that IL-17A does not decrease GRE transcriptional activity nor the translocation of the ligated receptor within 2 hours of incubation, the time frame in which IL-17A induced GC-sensitivity. Therefore, IL-17A-induced GC-insensitivity is not likely caused by a decrease in translocation of the ligated receptor, but the reduced HDAC activity appears to be the main contributor to this effect.

Discussion

There is increasing evidence that Th17 cells play a role in the development of GC-insensitive airway inflammation in asthma, although it is still poorly understood why Th17-dependent inflammation is less responsive to GCs. Here, we show that IL-17A induces GC-insensitivity of TNF-induced IL-8 production in human bronchial epithelial cells. Furthermore, our data demonstrate that IL-17A-induced IL-8 production is normally sensitive to GCs and that IL-17A does not induce GC-insensitive transcription, as previously suggested (8;14). We are the first to show that IL-17A-induced GC-insensitivity is mediated by PI3K-downstream signalling and a reduction in HDAC activity. As a result, this leads to impaired ability of GCs to efficiently suppress IL-8 production upon stimulation with pro-inflammatory cytokines (e.g. TNF-α). In contrast to PI3K downstream signalling, the MEK/ERK, p38 MAPK pathways and/or decreased GR translocation to the nucleus do not seem to play a role in IL-17A-induced GC-insensitivity in bronchial epithelial cells.

Although inhaled GCs are currently the most effective anti-inflammatory treatment of asthma, a subset of patients suffers from a difficult-to-treat and relatively GC-insensitive asthma, which is a burdensome problem in the management of asthma. The mechanisms underlying the poor response in this subset of patients are still largely unknown. Previous studies have shown a link between neutrophilic inflammation and GC-insensitivity in asthma (4;5) as well as between neutrophilic inflammation and the presence of Th17 cells in the airways (6). This led to the hypothesis that Th17-mediated inflammation related to GC-insensitivity. In line with this hypothesis, McKinley *et al* showed that the transfer of Th17 cells in mice results in GC-insensitive airway inflammation and airway hyperresponsiveness when compared to the inflammation induced upon the transfer of Th2 cells (7). Thus, Th17

cells have an emerging role in GC-insensitive neutrophilic inflammation, although the underlying mechanism of GC-insensitivity has remained unknown so far.

Our results demonstrate that Th17-induced GC-insensitivity is not mediated by activation of GC-insensitive pathways by IL-17A. Similar effects were observed by Prause *et al* (15), showing that IL-17A-induced production of IL-8 as well as GCP-2, GRO-α in 16HBE cells is sensitive to hydrocortisone. In contrast to Prause's and our findings, Jones *et al* (8) showed that IL-17A-induced IL-8 production is insensitive to dexamethasone in primary bronchial epithelial cells. One of the dissimilarities is that Jones *et al* seem to have conducted experiments under subconfluent conditions. However, a relation between cell-cell contacts and the actions of GCs has to our knowledge not been described. Another important difference is that Jones *et al* performed their experiments in primary epithelial cells, while we and Prause have used cell lines. In future studies we plan to compare the effects in cell lines to primary cells, although it may be of concern that the GC hydrocortisone is customary present in the culture medium of for primary cells.

We do not consider it likely that IL-17A plays a major role in the release of IL-8 in the airways and subsequent development of neutrophilia, since IL-17A was relatively inefficient in inducing IL-8 production when compared to the pro-inflammatory cytokine, TNF-α. Thus, we hypothesized that IL-17A exerts a different role in GC-sensitivity, reducing the responsiveness of IL-8 transcription to GCs.

In line with this hypothesis, we show that the pre-incubation with IL-17A reduces the capacity of GCs to inhibit TNF-α-induced IL-8 production. Thus, the presence of IL-17A secreting cells in the airways may render airway epithelial production of IL-8 less responsive to GCs. It is of importance to note that we did not observe an effect on the inhibitory effect of budesonide at high concentrations. One of

the explanations for the lack of effect on the maximum inhibition of budesonide is that IL-17A did not affect GR binding to the GRE region. Possibly, the inhibitory effect of GR binding to the IL-8 promoter predominates over the effect of HDAC2 on IL-8 production at higher concentrations of GCs. In addition to our novel findings on the role of IL-17A in GC-insensitivity, earlier studies concerning cytokine-induced GC-insensitivity have shown possible roles for different cytokines in GC-insensitivity, i.e. IL-2 in combination with IL-4 in T-cells (16), IL-13 in monocytes (17), TNF-α in combination with IFN-γ in airway smooth muscle cells (18) and IL-27 in combination with IFN-γ in macrophages (19). However, none of these cytokines can explain the association between Th17 cells and GC-insensitivity and clinical data on the association between these cytokines and GC-insensitivity in asthma is rather scarce. Since IL-17A induces GC-insensitivity of IL-8 production in epithelial cells, we postulate that IL-17A itself plays an important role in the development of GC-insensitive neutrophilic inflammation.

We show that IL-17A-induced PI3K/Akt signalling is involved in the observed effects on GC-sensitivity. IL-17A has previously been shown to activate PI3K signalling (20). Furthermore, PI3K activation has been implicated in H_2O_2- and cigarette smoke-induced GC-insensitivity in A549 cells, i.e. by a mechanism involving HDAC2 phosphorylation and subsequent inactivation (12). In line with this, we found that IL-17A activated the PI3K-downstream signalling and decreased overall HDAC activity. Specific overexpression of HDAC2 abrogated IL-17A-induced GC-insensitivity, strongly suggesting that PI3K activation and subsequent HDAC2 inactivation play a role in IL-17A-induced GC-insensitivity.

In addition to the PI3K-signaling pathway, IL-17A has been described to activate the MEK/ERK-signalling in A549 cells and primary tracheal cells (21;22),

and both the p38 MAPK and the MEK/ERK signalling in 16HBE cells (23). We confirmed this in our cells. Activation of the MEK/ERK and p38 pathways has been shown to induce phosphorylation of the GR and decreasing its translocation to the nucleus causing GC-insensitivity in various celltypes (13;24;25). However, our data do not support a role for the MEK/ERK and p38 MAPK pathways in IL-17A-induced GC-insensitivity in airway epithelial cells, since specific inhibition of these pathways could not block the IL-17A-induced effects. Moreover, GRE-mediated transcription was not impaired upon IL-17A exposure in airway epithelium, suggesting that IL-17A-induced MAPK activity does not result in altered translocation of the ligated receptor.

Previous studies in PBMCs and BAL fluid of asthmatic patients have suggested that GC-insensitivity in asthma patients is associated with increased GR-β (26;27), which acts as a negative competitor to the active variant GR-α. In line with these findings, Vazquez-Tello *et al* recently demonstrated that IL-17A induces GR-β (14). However, this does not likely contribute to the effect observed in our study, since we observed the strongest effect of IL-17A on GC-sensitivity already within 2 hours of pre-incubation, whereas Vazquez-Tello *et al* showed that IL-17A does not induce GR-β mRNA until 6 hours in primary bronchial epithelial cells. Moreover, if GR-β would play a role in our model, we would expect an effect on the transactivation of the GRE reporter construct due to the competitive nature of this mechanism. Since we did not observe such an effect, we do not render it likely that GR-β plays a role in our experiments.

Together with Vazquez-Tello *et al*, we are first to describe mechanisms of GC-insensitivity in bronchial epithelial cells. Previous studies on GC-insensitivity have mainly focused on leukocytes. However, bronchial epithelial cells have an

emerging role in the innate immune response and we speculate that changes in sensitivity to GCs in airway epithelial cells strongly contributes to GC-insensitivity of inflammatory responses in the lung.

Cigarette smoking has been associated with a poor response to GCs in asthma (28) and smoking asthmatics displayed increased levels of sputum neutrophils (29). Interestingly, IL-17A is increased in the BAL of a mouse model of cigarette smoke-induced inflammation (30;31), and therefore it is tempting to speculate that Th17 cells play a role in smoking induced GC-insensitivity as well. Strikingly, similar mechanisms have been proposed in smoking-induced GC-insensitivity, i.e. PI3K-dependent HDAC2 inactivation (32). Therefore, it will be of interest to study whether cigarette smoke-induced GC-insensitivity is (partly) mediated by IL-17A in future studies.

Given our novel results on the role of PI3K in GC-insensitivity, we propose that inhibition of the PI3K pathway may be a promising tool improve GC function and revert GC-insensitivity. In macrophages it has been demonstrated that theophylline treatment restores response to GCs by its inhibitory effect on PI3K activity (33). Therefore, we anticipate that treatment with theophylline might serve as a strategy to revert GC-insensitivity associated with IL-17A, especially given the fact that inhibition of PI3K was able to restore GC-sensitivity in our study as well. In asthma as well as COPD, clinical pilot studies have been performed on the effect of theophylline combined with inhaled GCs (34;35). Though these first data show some improvement in lung function, the authors of both studies also stress the need for more extensive investigation into this possible treatment, because both studies were relatively small and single-centred. No *in vivo* and clinical data are present on the

action of theophylline on Th17 mediated inflammation and the associated steroid insensitivity in severe asthma, so further studies are warranted.

In conclusion, we demonstrate that IL-17A is able to induce GC-insensitivity in bronchial epithelial cells by activating the PI3K pathway, which may lead to a decrease in HDAC2 activity. Therefore, we propose that therapeutic strategies to inhibit PI3K as well as therapies focused on downregulating Th17 activity and secretion of IL-17A may lead to novel ways to improve the efficacy of GCs.

Figure legends

Figure 1. (A) IL-17A induces IL-8 production in 16HBE cells. Cells were serum-deprived overnight, washed and treated for 24h with the indicated amounts of IL-17A. (B) IL-17A-induced IL-8 production is dose-dependently inhibited by budesonide in 16HBE cells. Cells were pre-treated with control medium, 10^{-11} M budesonide or 10^{-9} M budesonide for 2h, washed and treated for 24h with 10 ng/ml IL-17A. Absolute values of IL-8 ± SEM are shown in pg/ml (n=3). **=$p<0.01$ between the indicated values.

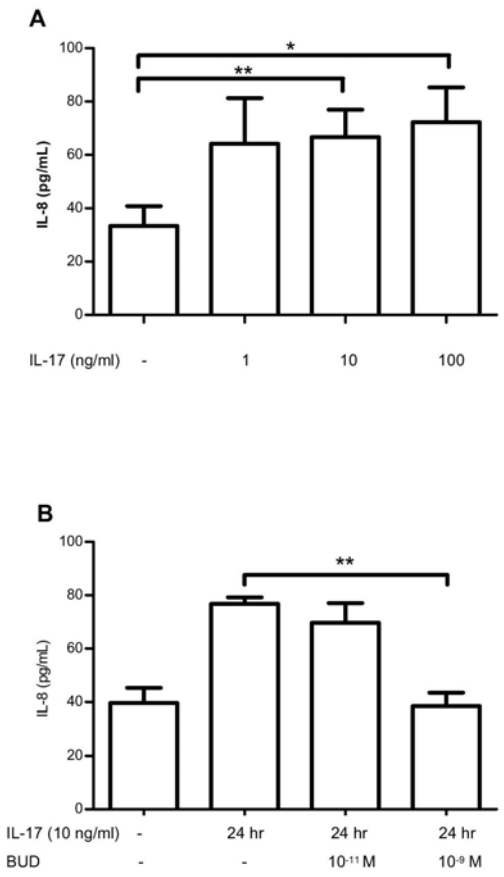

Figure 1

Figure 2. Inhibition of TNF-α-induced IL-8 production by budesonide is impaired by pre-incubation with IL-17A. Cells were serum-deprived overnight, pre-treated with 10 ng/ml IL-17A (black bars) or control medium (white bars) for 2h, washed, treated for 2h with control medium or budesonide, washed, and finally incubated for 24h with 10

ng/ml TNF-α. Absolute values of IL-8 ± SEM are shown in pg/ml (n=4) **=p<0.01 between the indicated values.

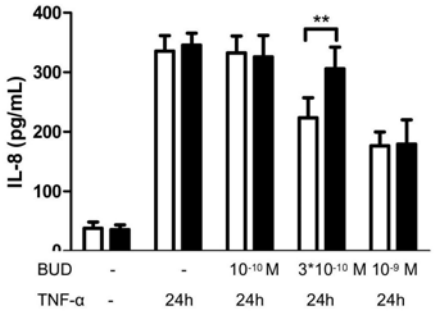

Figure 2

Figure 3. (A) IL-17A induces phosphorylation of ERK, p38 and Akt. Cells were serum-deprived overnight. IL-17A was added for the indicated period. Phosphorylation of ERK, p38 and Akt was assessed by immunodetection. Representative blots of 4 independent experiments are shown. (B-D) Analysis by densitometry of immunodetection of p-ERK (B), p-Akt (C) and (D) p-p38 (n=4) **p<0.01 ***p<0.001 (~~B~~E)Inhibitor of PI3K negates the effects of IL-17A on inhibition of IL-8 production by budesonide. 30 min prior to IL-17A treatment, the PI3K, ERK and p38 inhibitors were added: LY294002 (10 μM), U0126 (10 μM) and SB203580 (1 μM), respectively. 10 ng/ml of IL-17A was added and cells were incubated for 2h, washed incubated for 2h with $3*10^{-10}$ M budesonide, washed and then incubated for 24h with 10 ng/ml TNF-α. Cell-free supernatants were harvested and IL-8 was determined with ELISA. Data are shown as percentage ± SEM of inhibition by budesonide of corresponding TNF-α stimulated control (n=4). White bar is treated with control, black bar with IL-17A, grey bars are treated with specific pathway inhibitors prior to IL-17A. n=4 *=p<0.05

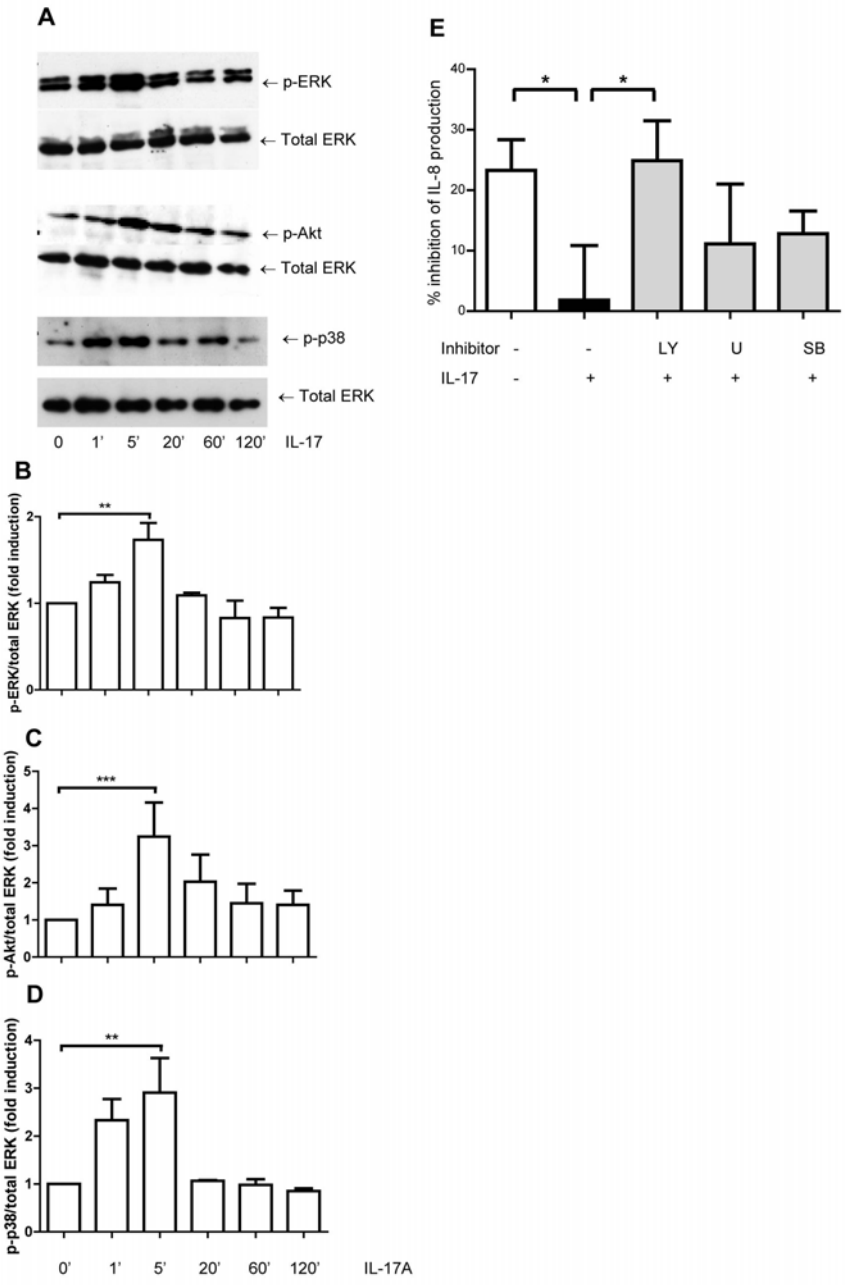

Figure 3

Figure 4. (A) IL-17A decreases HDAC activity. Cells were serum-deprived overnight, treated for 2h with (black bar) and without (white bar) 10 ng/ml IL-17A, resuspended in RIPA-buffer and sonicated. HDAC activity was measured using a fluorometric activity assay. HDAC activity is shown as a ratio of the fluorescent units

per milligram of protein(n=4). *=p<0.05 (B) Effect of HDAC2 overexpression on IL-17A-induced GC-insensitivity. Cells were grown to 90-95% confluency before transfection with control vector or HDAC2 overexpression (grey bar). Cells were then pre-treated with 10 ng/ml IL-17A (black bar) or control medium (white bar) for 2h, washed, treated for 2h with control medium or budesonide, washed, and finally incubated for 24h with 10 ng/ml TNF-α. Cell-free supernatants were harvested and IL-8 was determined with ELISA. Data are shown as percentage± SEM of inhibition by budesonide of corresponding TNF-α stimulated control (n=~~4~~8) *=p<0.05; **=p<0.01.

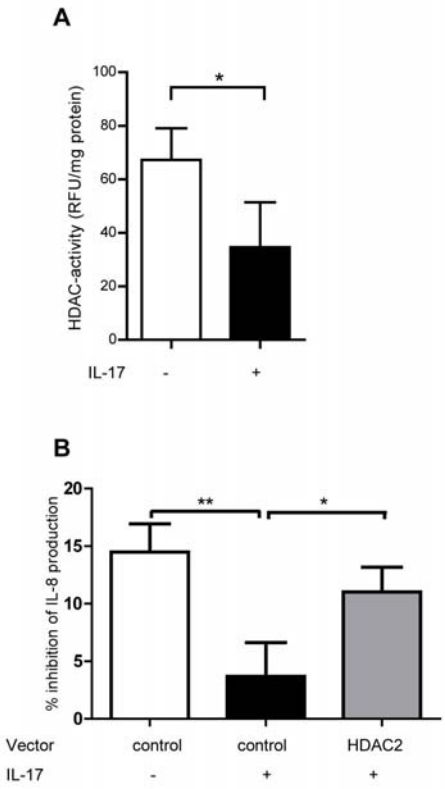
A
HDAC-activity (RFU/mg protein)
100
80
60
40
20
0
*
IL-17
-
+
B
% inhibition of IL-8 production
20
15
10
5
0
**
*
Vector
control
control
HDAC2
IL-17
-
+
+

Figure 4

Figure 5. IL-17A has no effect on GRE-mediated transcription. Cells were grown to 90-95% confluency before transfection with GRE driven Firefly luciferase with Renilla luciferase as a control for transfection efficiency. Cells were pre-treated for 2h with (black bars) or without (white bars)10 ng/ml IL-17A, treated for 24h with budesonide (concentrations 10^{-9}-$3*10^{-8}$), resuspended in lysis buffer and activity was assessed for both luciferases. Data is shown as fold induction ± SEM over control (n=4).

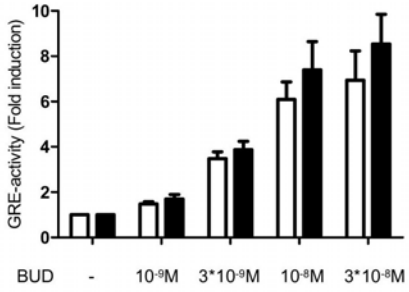
GRE-activity (Fold induction)
10
8
6
4
2
0
BUD
-
10-9M
3*10-9M
10-8M
3*10-8M

Figure 5

Reference List

(1) Carmichael J, Paterson IC, Diaz P, Crompton GK, Kay AB, Grant IW. Corticosteroid resistance in chronic asthma. Br Med J (Clin Res Ed) 1981 May 2;282(6274):1419-22.

(2) Schwartz HJ, Lowell FC, Melby JC. Steroid resistance in bronchial asthma. Ann Intern Med 1968 Sep;69(3):493-9.

(3) Barnes PJ, Adcock IM. Glucocorticoid resistance in inflammatory diseases. Lancet 2009 May 30;373(9678):1905-17.

(4) Cowan DC, Cowan JO, Palmay R, Williamson A, Taylor DR. Effects of steroid therapy on inflammatory cell subtypes in asthma. Thorax 2010 May;65(5):384-90.

(5) Little SA, Chalmers GW, MacLeod KJ, McSharry C, Thomson NC. Non-invasive markers of airway inflammation as predictors of oral steroid responsiveness in asthma. Thorax 2000 Mar;55(3):232-4.

(6) Alcorn JF, Crowe CR, Kolls JK. TH17 Cells in Asthma and COPD. Annual Review of Physiology 2010 Mar 1;72(1):495-516.

(7) McKinley L, Alcorn JF, Peterson A, Dupont RB, Kapadia S, Logar A, et al. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. J Immunol 2008 Sep 15;181(6):4089-97.

(8) Jones CE, Chan K. Interleukin-17 stimulates the expression of interleukin-8, growth-related oncogene-alpha, and granulocyte-colony-stimulating factor by human airway epithelial cells. Am J Respir Cell Mol Biol 2002 Jun;26(6):748-53.

(9) Vanaudenaerde BM, Wuyts WA, Dupont LJ, Van Raemdonck DE, Demedts MM, Verleden GM. Interleukin-17 stimulates release of interleukin-8 by human airway smooth muscle cells in vitro: a potential role for interleukin-17 and airway smooth muscle cells in bronchiolitis obliterans syndrome. J Heart Lung Transplant 2003 Nov;22(11):1280-3.

(10) Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. Thorax 2002 Oct;57(10):875-9.

(11) Heijink IH, Kies PM, Kauffman HF, Postma DS, van Oosterhout AJ, Vellenga E. Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity. J Immunol 2007 Jun 15;178(12):7678-85.

(12) Moodie FM, Marwick JA, Anderson CS, Szulakowski P, Biswas SK, Bauter MR, et al. Oxidative stress and cigarette smoke alter chromatin remodeling but differentially regulate NF-kappaB activation and proinflammatory cytokine release in alveolar epithelial cells. FASEB J 2004 Dec;18(15):1897-9.

(13) Irusen E, Matthews JG, Takahashi A, Barnes PJ, Chung KF, Adcock IM. p38 Mitogen-activated protein kinase-induced glucocorticoid receptor phosphorylation reduces its activity: role in steroid-insensitive asthma. J Allergy Clin Immunol 2002 Apr;109(4):649-57.

(14) Vazquez-Tello A, Semlali A, Chakir J, Martin JG, Leung DY, Eidelman DH, et al. Induction of glucocorticoid receptor-beta expression in epithelial cells of asthmatic airways by T-helper type 17 cytokines. Clinical & Experimental Allergy 2010;40(9):1312-22.

(15) Prause O, Laan M, Lotvall J, Linden A. Pharmacological modulation of interleukin-17-induced GCP-2-, GRO-alpha- and interleukin-8 release in human bronchial epithelial cells. Eur J Pharmacol 2003 Feb 21;462(1-3):193-8.

(16) Kam JC, Szefler SJ, Surs W, Sher ER, Leung DY. Combination IL-2 and IL-4 reduces glucocorticoid receptor-binding affinity and T cell response to glucocorticoids. J Immunol 1993 Oct 1;151(7):3460-6.

(17) Spahn JD, Szefler SJ, Surs W, Doherty DE, Nimmagadda SR, Leung DY. A novel action of IL-13: induction of diminished monocyte glucocorticoid receptor-binding affinity. J Immunol 1996 Sep 15;157(6):2654-9.

(18) 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 Feb;69(2):588-96.

(19) Li JJ, Wang W, Baines KJ, Bowden NA, Hansbro PM, Gibson PG, et al. IL-27/IFN-gamma induce MyD88-dependent steroid-resistant airway hyperresponsiveness by inhibiting glucocorticoid signaling in macrophages. J Immunol 2010 Oct 1;185(7):4401-9.

(20) Huang F, Kao CY, Wachi S, Thai P, Ryu J, Wu R. Requirement for both JAK-mediated PI3K signaling and ACT1/TRAF6/TAK1-dependent NF-kappaB activation by IL-17A in enhancing cytokine expression in human airway epithelial cells. J Immunol 2007 Nov 15;179(10):6504-13.

(21) Ning W, Choi AM, Li C. Carbon monoxide inhibits IL-17-induced IL-6 production through the MAPK pathway in human pulmonary epithelial cells. Am J Physiol Lung Cell Mol Physiol 2005 Aug;289(2):L268-L273.

(22) Inoue D, Numasaki M, Watanabe M, Kubo H, Sasaki T, Yasuda H, et al. IL-17A promotes the growth of airway epithelial cells through ERK-dependent signaling pathway. Biochem Biophys Res Commun 2006 Sep 8;347(4):852-8.

(23) Laan M, Lotvall J, Chung KF, Linden A. IL-17-induced cytokine release in human bronchial epithelial cells in vitro: role of mitogen-activated protein (MAP) kinases. Br J Pharmacol 2001 May;133(1):200-6.

(24) Tsitoura DC, Rothman PB. Enhancement of MEK/ERK signaling promotes glucocorticoid resistance in CD4+ T cells. J Clin Invest 2004 Feb;113(4):619-27.

(25) Onda K, Nagashima M, Kawakubo Y, Inoue S, Hirano T, Oka K. Mitogen-activated protein kinase kinase 1/extracellular signal-regulated kinase (MEK-1/ERK) inhibitors sensitize reduced glucocorticoid response mediated by TNFalpha in human epidermal keratinocytes (HaCaT). Biochem Biophys Res Commun 2006 Dec 8;351(1):266-72.

(26) Leung DY, Hamid Q, Vottero A, Szefler SJ, Surs W, Minshall E, et al. Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. J Exp Med 1997 Nov 3;186(9):1567-74.

(27) Goleva E, Li LB, Eves PT, Strand MJ, Martin RJ, Leung DY. Increased glucocorticoid receptor beta alters steroid response in glucocorticoid-insensitive asthma. Am J Respir Crit Care Med 2006 Mar 15;173(6):607-16.

(28) Thomson NC, Chaudhuri R, Livingston E. Asthma and cigarette smoking. Eur Respir J 2004 Nov;24(5):822-33.

(29) Chalmers GW, MacLeod KJ, Thomson L, Little SA, McSharry C, Thomson NC. Smoking and airway inflammation in patients with mild asthma. Chest 2001 Dec;120(6):1917-22.

(30) van der Deen M, Timens W, Timmer-Bosscha H, van der Strate BW, Scheper RJ, Postma DS, et al. Reduced inflammatory response in cigarette smoke exposed Mrp1/Mdr1a/1b deficient mice. Respir Res 2007;8:49.

(31) Melgert BN, Timens W, Kerstjens HA, Geerlings M, Luinge MA, Schouten JP, et al. Effects of 4 months of smoking in mice with ovalbumin-induced airway inflammation. Clin Exp Allergy 2007 Dec;37(12):1798-808.

(32) Marwick JA, Caramori G, Casolari P, Mazzoni F, Kirkham PA, Adcock IM, et al. A role for phosphoinositol 3-kinase [delta] in the impairment of glucocorticoid responsiveness in patients with chronic obstructive pulmonary disease. Journal of Allergy and Clinical Immunology 2010 May;125(5):1146-53.

(33) To Y, Ito K, Kizawa Y, Failla M, Ito M, Kusama T, et al. Targeting Phosphoinositide-3-Kinase-{delta} with Theophylline Reverses Corticosteroid Insensitivity in Chronic Obstructive Pulmonary Disease. Am J Respir Crit Care Med 2010 Oct 1;182(7):897-904.

(34) Spears M, Donnelly I, Jolly L, Brannigan M, Ito K, McSharry C, et al. Effect of low-dose theophylline plus beclometasone on lung function in smokers with asthma: a pilot study. Eur Respir J 2009 May;33(5):1010-7.

(35) Ford PA, Durham AL, Russell REK, Gordon F, Adcock IM, Barnes PJ. Treatment Effects of Low-Dose Theophylline Combined With an Inhaled Corticosteroid in COPD. Chest 2010 Jun 1;137(6):1338-44.