**Inhibition of PI3Kδ restores glucocorticoid function in smoking-induced airway inflammation in mice**

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**Scientific Knowledge on the Subject:** Glucocorticoid unresponsiveness in severe asthma COPD may involve an oxidant mediated impairment of glucocorticoid receptor alpha (GRα) function through reduction of histone deacetylase activity and co-repressor expression.

**What This Study Adds to the Field:** Histone deacetylase 2 activity is reduced in smoke exposed mice lungs correlating with reduced glucocorticoid function which is restored by PI3Kδ but not γ inhibition. GRα expression also is reduced in smoke exposed mouse and in COPD patient lungs.
Abstract

Rational: There is an increasing prevalence of reduced responsiveness to glucocorticoid therapy in severe asthma and chronic obstructive pulmonary disease, however the molecular mechanism of this remains unknown. Recent studies have shown that histone deacetylase activity, which is critical to glucocorticoid function, is altered by oxidant stress and may be involved in the development of glucocorticoid insensitivity.

Objectives: To determine the role of phosphoinositol-3-kinase (PI3K) in the development of cigarette smoke induced glucocorticoid insensitivity.

Methods: Wild type, PI3Kγ knock-out and PI3Kδ kinase dead knock-in transgenic mice were used in a model of cigarette smoke induced glucocorticoid insensitivity. Peripheral lung tissue was obtained 6 healthy non-smokers, 9 smokers with normal lung function and 8 patients with chronic obstructive pulmonary disease.

Measurements and Main Results: Glucocorticoid receptor expression was significantly reduced in both the lungs of chronic obstructive pulmonary disease patients and in cigarette smoke-exposed mice. Furthermore, cigarette smoke exposure in mice increased tyrosine nitration of histone deacetylase 2 in the lung correlating with both reduced histone deacetylase 2 activity and reduced glucocorticoid function. Oxidative stress activated Akt and induced glucocorticoid insensitivity in vitro, which was restored by inhibition of PI3K. In vivo, histone deacetylase 2 activity and the anti-inflammatory effects of glucocorticoids were restored in PI3Kδ kinase dead knock-in but not PI3Kγ knock-out smoke exposed mice compared to wild types, correlating with reduced histone deacetylase 2 tyrosine nitration.

Conclusion: Together these data shows that therapeutic inhibition of PI3Kδ may restore glucocorticoid function in oxidative stress induced glucocorticoid insensitivity.

Abstract Word count: 247

Key Words: inflammation, histone deacetylase, chromatin, oxidative stress
Introduction

Glucocorticoids are ineffective in severe asthma and chronic obstructive pulmonary disease (COPD), even at high oral doses thereby presenting considerable disease management problems and cost burden with few effective alternative treatments (1,2). Both these conditions have a strong component of oxidative stress which may contribute to the development of this apparent glucocorticoid unresponsiveness, however, the precise molecular mechanism(s) of this apparent impairment remains unclear.

The human GR gene encodes two isoforms; GRα, through which the actions of glucocorticoids are mediated, and the non glucocorticoid binding GRβ (3). The major glucocorticoid anti-inflammatory action is mediated by transrepression of pro-inflammatory genes (4). Here, GRα monomers associate with promoter bound transcription factors such as nuclear factor NF-κB and AP-1 (5) followed by recruitment ‘co-repressor complexes’ such as mSin3a (mammalian Sin3a) and Mi-2α/β (chromodomain helicase DNA binding protein) (6-8). These act as ‘scaffold proteins’ by assembling multiple components including histone deacetylase (HDAC) activity, and critically HDAC-2 (9,10), which is recruited to remove the acetyl moieties from the amino terminal (NH) tails of the core histones at the promoter sight of transcriptional active genes. This allows ‘re-condensation’ of the DNA around the core histone proteins, thereby dislodging the transcriptional machinery leading to cessation of gene transcription (11). HDAC-2 is also implicated in deacetylation of other transcriptional regulators including GRα itself, thereby allowing NF-κB binding and subsequent glucocorticoid-mediated transrepression of NF-κB dependant gene expression (10). Indeed, in relative glucocorticoid insensitive conditions including severe asthma and COPD, HDAC-2 expression is reduced, correlating with increased pro-inflammatory cytokine release, relative glucocorticoid insensitivity and disease severity (12-14). Furthermore, knock-down of HDAC-2 expression in bronchoalveolar (BAL) macrophages induces a relative glucocorticoid
insensitivity and conversely overexpression of HDAC-2 in BAL macrophages from patients with COPD restores glucocorticoid function (10).

The bronchodilator theophylline acts as a glucocorticoid sparing agent in asthmatics (15) and has can restore glucocorticoid function in alveolar macrophages from COPD patients in vitro, corresponding with restored HDAC-2 activity (16;17), however the mechanism remains unclear. One of the potential targets of theophylline is the lipid kinase phosphoinositol-3-kinase (PI3K) (18). Furthermore, both PI3Kδ and γ are proposed as potential anti-inflammatory targets (19;20) with PI3Kδ implicated in B- and T- cell signalling, mast cell mediated allergic response and neutrophil activation and PI3Kγ linked to neutrophil activation, mast cell degranulation and T cell function (21-24).

Here we investigate the molecular mechanism of glucocorticoid insensitivity. Using transgenic mice in an animal model of cigarette smoke-induced glucocorticoid resistance we show a reduction in HDAC-2 activity correlating with reduced glucocorticoid function which is restored by PI3Kδ but not γ inhibition with further alterations in lung mSin3a and Mi-2α/β expression and a reduction in lung GRα expression. Furthermore, both GRα and GRβ expression is reduced in COPD patient lungs.

**Methods**

**Cell culture & Treatments.** U937 cells were cultured in RPMI 1640 GlutaMAX media with 10% and BEAS-2B cells were cultured in Keratinocyte media with LG and supplements for K-SFM. All cell culture reagents were purchased from Invitrogen (Paisley, UK), unless otherwise stated. **Reagents:** H2O2 (Sigma Dorset, UK), LY294002 (Merck Biosciences, Nottingham, UK), buesonide (Sigma) and TNFα (R&D Systems, Abingdon, UK). **Antibodies:** Akt (New England BioLabs, Herts, UK); HDAC-2, GRα, lamin A/C, actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mSin3a, GAPDH, Nitrotyrosine and
phosphoserine (Abcam, Cambridge, UK); GRβ (Affinity Bioreagents, CO, USA); Mi-2α/β (Austral Biologicals, San Ramon, CA, USA).

**Cigarette smoke induced GC insensitive mouse model:** Studies described herein were performed under a Project License issued by the United Kingdom Home Office and protocols were approved by the Local Ethical Review Process. Both PI3Kγ−/− and PI3KδD910A/D910A mice have been described previously (25;26). Mice were exposed to either cigarette smoke (5x1R3F cigarettes/day) or room-air on 3 consecutive days as previously described (27) and dosed with either budesonide (1mg/kg) or vehicle (saline with 2% NMP) by intranasal (i.n.) administration one hour prior to exposure. Animals were sacrificed 24 hours with bronchiolar lavage and differential cell counts performed as previously described (27).

**Protein extraction, Immunoblotting and Immunoprecipitation.** Cytosolic proteins were extracted using a hypotonic lysis buffer (10mM Tris HCl pH6.5, 0.5mM Na Bisulfite, 10mM MgCl₂, 8.6% sucrose, 0.5% NP-40 phosphatase inhibitors and protease inhibitors). Nuclear proteins were extracted using a high salt extraction buffer (15mM Tris HCL pH 7.9, 450mM NaCl, 10% glycerol, phosphatase inhibitors and protease inhibitors) and nuclear extract salt concentrations normalised with 2 volumes of a Tris-glycerol buffer (15mM Tris HCL pH 7.9, 10% glycerol, phosphatase inhibitors and protease inhibitors). Protein quantification was assessed by BCA assay (Perbio, Northumberland, UK). Immunoblotting and immunoprecipitation was performed as previously described (28).

**ELISA & HDAC activity assay.** KC and IL-6 and IL-8 levels were measured using Quantikine ELISA kits (RnD Systems), and HDAC activity was measure by HDAC activity assay kit (Biomol International, PA, USA) according to manufacturer’s instructions.
**Human study subjects.** All subjects were recruited from the Section of Respiratory Medicine of the University Hospital of Ferrara, Italy with approval by the local ethics committee of the University Hospital of Ferrara (table 1). All the subjects were free from bronchodilator, theophylline, antibiotic, antioxidant and/or glucocorticoid therapy in the last month before surgery. Pulmonary function tests were performed as previously described (29). COPD and chronic bronchitis were respectively defined, according to international guidelines, as the presence of post-bronchodilator FEV\textsubscript{1}/FVC ratio<70% or the presence of cough and sputum production for at least 3 months in each of two consecutive years (30). Lung tissue processing and immunohistochemistry was performed as previously described (29).

**Statistical analysis.** For all experiments, the statistical significance of differences between samples was calculated on GraphPad Prism software using Students t-test (Mann-Whitney test). Data is expressed as mean ± SEM, differences were considered significant if \( P < 0.05 \).

**Results**

**Oxidative stress induces Akt phosphorylation and reduced glucocorticoid function in vitro.** Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) induced Akt phosphorylation in a time and PI3K-dependant manner (Fig. 1A). H\textsubscript{2}O\textsubscript{2} exposure alone only induced a small 0.5-1 fold increase in IL-8 release, but augmented the levels of IL-8 release induced by TNF\( \alpha \) (Fig. 1B). Pre-treatment with 100nM dexamethasone gave a maximal inhibition of TNF\( \alpha \) mediated IL-8 release (~50-60%), however in cells exposed to H\textsubscript{2}O\textsubscript{2}, 100nM dexamethasone was only able to reduced TNF\( \alpha \) induced IL-8 levels to that comparable to TNF\( \alpha \) alone (Fig. 1B). Inhibition of PI3K restored dexamethasone suppression of IL-8 release with no apparent impact alone (Fig. 1C).
Inhibition of PI3Kδ enables glucocorticoid suppression of cigarette smoke-induced lung inflammation. BAL neutrophil counts (Fig. 2) and whole lung tissue levels of the pro-inflammatory cytokines keratinocyte-derived chemokine (KC) and IL-6 (Fig. 3A, B) were measured as markers of cigarette smoke induced lung inflammation. Cigarette smoke exposure induced a marked inflammatory response in WT mice which budesonide failed to reduce, confirming this as a model of glucocorticoid insensitivity. There were no significant differences in either neutrophil number (Fig. 2) or cytokine lung tissue levels (Fig. 3A, B) between the wild type BALB/c (WT) and PI3Kδ kinase dead knock-in PI3KδD910A/D910A or PI3Kγ knockout PI3Kγ−/− sham exposed mice. Cigarette smoke exposure resulted in both an influx of neutrophils into the lung and increased KC and IL-6 lung tissue levels. Budesonide treatment at 1mg/kg failed to reduce either the neutrophil influx or tissue cytokine levels in the WT mice, confirming glucocorticoid insensitivity in this model (Fig. 3A, B). However, budesonide treatment in PI3KδD910A/D910A mice, but not PI3Kγ−/− reduced both the neutrophil influx and lung tissue cytokine levels, indicating that the PI3Kδ pathway may play a role in the development of cigarette smoke induced glucocorticoid insensitivity.

Inhibition of PI3Kδ restores HDAC activity after cigarette smoke exposure in vivo. Oxidative stress can impair HDAC-2 activity which is implicated in the development of glucocorticoid insensitivity. Consistent with this, total nuclear HDAC and nuclear HDAC-2 activity was reduced in WT and PI3Kγ−/− mice lungs (Fig. 4A, B). However, in smoke exposed PI3KδD910A/D910A mice, both total nuclear and nuclear HDAC-2 activity was unaffected (Fig. 4A, B). Budesonide treatment had no significant effect on either total nuclear HDAC or HDAC-2 activity. There was no difference in nuclear HDAC-2 expression levels between any of the groups (data not shown) indicating that the observed reduction in HDAC-
2 activity was due to reduced activity alone rather than a reduction in expression. To establish the cause of the reduced HDAC-2 activity, expression levels and post-translational modifications were assessed (Fig. 5). Assessment of immunoprecipitated HDAC-2 revealed that both tyrosine nitration and serine phosphorylation were elevated in the smoke exposed WT mice with no impact of PI3Kγ⁻/⁻ (Fig. 5A, B). However, both tyrosine nitration and serine phosphorylation of HDAC-2 were reduced in smoke exposed PI3KδD910A/D910A mice compared to WT controls (Fig. 5A, B). Budesonide treatment had no additional impact on either tyrosine nitration of serine phosphorylation in any group.

**Cigarette smoke exposure alters mSin3a and Mi-2α/β expression.** It is possible that the cigarette smoke-induced reduction in HDAC-2 activity may relate to changes in the expression of other co-repressor components such as the ‘chaperone proteins’ mSin3a and Mi-2α/β which co-ordinate and orchestrate the HDAC-2 co-repressor complex. Both mSin3a and Mi-2α expression was reduced in cigarette smoke exposed mice with no impact of either PI3Kδ or γ inhibition (Fig. 6A, B). Interestingly, budesonide treatment prevented the reduction of mSin3a expression in all groups (Fig. 6A), however Mi-2α expression was only maintained in the budesonide treated PI3Kγ⁻/⁻, PI3KδD910A/D910A mice (Fig. 6B). Conversely, Mi-2β expression was elevated in all smoke exposed groups with no discernable difference between PI3Kδ or γ inhibition or any further impact by budesonide treatment (Fig. 6C).

**GR expression is reduced by cigarette smoke and in COPD peripheral lung tissue.** The reduction in glucocorticoid function in the smoke exposed mice may also be due to an alteration in the expression and/or translocation of GRα itself. There was no difference in GRα protein expression between WT and PI3Kγ⁻/⁻ or PI3KδD910A/D910A sham mice in either the cytosolic or nuclear compartments. Cigarette smoke exposure significantly reduced GRα
protein expression with no impact of either PI3Kδ or γ inhibition (Fig. 7 A, B). However, there was also no difference in the GRα cytosolic:nuclear ratios in the smoke exposed mice with and without budesonide treatment (data not shown) indicating that budesonide mediated GRα translocation is ineffective in smoke exposed animals. To assess if this reduction in GRα expression was translated in humans in an oxidant driven glucocorticoid insensitive disease, peripheral lung from COPD patients, age matched normal subjects and smokers with normal lung function was assessed (Table 1). Immunohistochemical analysis demonstrated GRα staining of the bronchiolar and alveolar epithelial cells, bronchiolar smooth muscle cells, endothelial cells and infiltrating cells with no significant difference between nuclear and cytosolic localisation seen between COPD patients and the control groups (Fig. 8A-F). There was a significant reduction in the expression of both GRα and GRβ protein in the peripheral lung of COPD patients compared to age-matched normal subjects and smokers with normal lung function (Fig. 8G, H).

**Discussion**

We show here that oxidative stress results in loss of glucocorticoid function associated with increased post-translational modifications of HDAC-2 and subsequent reduction in HDAC-2 activity. Specific inhibition of PI3Kδ protected/restored HDAC activity correlating with attenuation of post-translational modifications and restored glucocorticoid function. Furthermore, we show that oxidative stress impacts on the GR/HDAC-2 co-complex-repressors mSin3a and Mi-2α/β. Although oxidative stress reduced GRα expression, restoration of GRα function in PI3Kδ<sup>D910/D910</sup> mice does not alter GR expression. Oxidative stress impaired glucocorticoid anti-inflammatory action and elevated Akt phosphorylation in a time and PI3K-dependant manner. Further preliminary data showed elevated Akt phosphorylation in peripheral blood mononuclear cells from patients with COPD.
compared with normal age-matched non-smokers (2.00±0.57 versus 1.14±0.21, data not shown). Interestingly, LY294002 restored glucocorticoid function in oxidative stressed cells whilst having no anti-inflammatory effect on its own. Indeed, cigarette smoke exposure induced a similar inflammatory response in the lungs of both PI3Kγ−/− and PI3KδD910A/D910A mice indicating that acutely, neither of these isoforms impact on oxidant induced inflammation. Contrary to this, both pharmacological and knock out studies show that PI3Kγ and δ inhibition is in itself anti-inflammatory (20-24;31-33). However, recent evidence suggests that an anti-inflammatory action in response to cigarette smoke may take weeks to develop, thus the 3 days of smoke exposure may not have been sufficient for any anti-inflammatory effect of PI3Kγ−/− or PI3KδD910A/D910A to be seen (34). Interestingly, budesonide pre-treatment had no impact on the inflammatory response in PI3Kγ−/− mice, but reduced the inflammatory response in PI3KδD910A/D910A mice indicating that oxidative induced glucocorticoid insensitivity may be linked to activation of PI3Kδ specific signalling. However the specific signalling pathways of the PI3Kδ isoforms remain unclear.

Smoke exposure also reduced both cytoplasmic and nuclear GR expression in the lung tissue with no apparent impact of PI3K inhibition. Interestingly, there was also no significant elevation of GRα levels in the nuclear compartment with budesonide, indicating that cigarette smoke exposure may impact on GRα translocation. However, as glucocorticoid function was restored in PI3KδD910A/D910A mice with no further impact on GRα expression or translocation, the endogenous levels of nuclear GRα may have been sufficient to provide relative restoration of the anti-inflammatory transrepression without elevated GRα translocation. This data therefore suggests that the reduction of GRα is unlikely to be the primary mechanism of glucocorticoid insensitivity in this model. Consistent with this model of cigarette smoke induced glucocorticoid insensitivity, assessment in clinical tissues revealed that GRα
expression is reduced in the periphery of the lung of smokers and is further reduce in the lungs from patients with the glucocorticoid-insensitive disease COPD. Furthermore, lung GRβ expression was also reduced in both smokers and COPD, indicating that GRβ is unlikely to play a significant role in the development of glucocorticoid insensitivity in COPD. 

HDAC-2 activity is critical to GRα transrepression and impaired HDAC-2 activity may be central in both glucocorticoid insensitivity in both severe asthma and COPD (10;12;13). Consistent with this, both total nuclear HDAC and nuclear HDAC-2 activity in the lung was reduced in smoke exposed mice. Interestingly however, inhibition of PI3Kδ but not PI3Kγ protected nuclear total HDAC and HDAC-2 activity, correlating with the glucocorticoid insensitivity in smoke exposed wt and PI3Kγ−/− but not PI3KδD910A/D910A mice. HDAC-2 expression itself remained unchanged in all groups indicating that reduction of activity must be post translational rather than an effect on expression per se. We have previously shown that HDAC-2 is subject to oxidative modifications which may in turn alter its activity and hyperphosphorylation is known to disrupt HDAC-2 interactions with other co-repressors (28;35;36). Indeed, both HDAC-2 tyrosine nitration and serine phosphorylation were elevated in smoke exposed animals and again, consistent with both the HDAC-2 activity and glucocorticoid function, only the PI3KδD910A/D910A mice had reductions in both tyrosine nitration and phosphorylation. This correlation between HDAC-2 activity, modifications and glucocorticoid function with PI3K inhibition may therefore provide a potential mechanism and therapeutic target for the restoration of glucocorticoid function.

Relatively little is known about either the composition or the stepwise construction/targeting of the GRα/HDAC-2 co-repressor complexes. The co-repressor ‘scaffold’ proteins mSin3a and Mi-2α/β are through to coordinate the construction of the co-repressor complexes to deliver both HDAC and methyl transference activity to the site of gene transcription (6-8). It is highly likely that oxidant stress induced alterations in these other co-repressor components,
potentially contributing to the mechanism of oxidative induced glucocorticoid insensitivity. Indeed, smoke exposure reduced both mSin3a and Mi-2α expression in the lung whilst Mi-2β was conversely elevated. Interestingly, glucocorticoid treatment elevated mSin3a expression independent of PI3Kγ/δ inhibition, whilst only restoring Mi-2α expression in mice with PI-3Kγ/δ inhibition. To our knowledge this is the first time that the direct impact oxidative stress on the expression of either mSin3a or Mi-2α/β has been studied and these alterations may further contribute to the mechanism of cigarette smoke mediated glucocorticoid insensitivity. Further investigation is needed to elucidate any functional and direct/indirect impact these and other changes in the co-repressor complexes induced by cigarette smoke have on oxidant induced corticosteroid insensitivity.

Although cigarette smoke exposure in animal models is often used to induce structural changes in the lung representative of emphysema-like pathology, the desired relative glucocorticoid-insensitivity for this study was achieved after a relatively acute exposure. Therefore no projection can be made as to the possible impact of PI3K isoform inhibition on structural changes induced by cigarette smoke and which are beyond the remit of this study. Combined this data shows that oxidative stress confers a relative glucocorticoid insensitivity in the airways during cigarette smoke-induced inflammation which is protected by specific inhibition of the PI3Kδ by a mechanism that involves the restoration of HDAC-2 activity. Furthermore, oxidant induced reduction of GRα expression, impaired translocation and alteration in mSin3a and M2α may limiting the level of nuclear GRα available for transrepression, thereby representing represent additional mechanisms by which oxidative stress impairs glucocorticoid sensitivity. Clinically, the development of PI3Kδ specific inhibitors may provide a means of overcoming the relative glucocorticoid insensitivity induced by oxidative stress in conditions such as COPD and severe asthma that affect millions of patients worldwide and whose current therapy is sub-optimal.
Reference List


phosphoinositide 3-kinase δ in promoting neutrophil trafficking into inflamed tissue.

_Blood_ 2004; _103_:3448-3456.


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Table 1: Characteristics of subjects for the study

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Age</th>
<th>Sex</th>
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<td>4 Ex : 4 Cur</td>
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<td>6 yes : 2 no</td>
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For COPD and smokers with normal lung function subjects FEV₁ %predicted and FEV₁/FVC% are post-bronchodilator values; data expressed as mean ± SEM.

* Forced expiratory volume in one second
† Forced vital capacity
‡ Non-Smoker
§ Male
** Female
†† Ex-smoker
‡‡ Current smoker
**Figure 4**

A

![Graph A](image1.png)

B

![Graph B](image2.png)

**Figure 5**

A

![Graph A](image3.png)

B

![Graph B](image4.png)
Figure 6

A

WT  PI3Kγ-/-  PI3Kγ-/-

Sham
Smoke (S)
S+Bud

Nuclear mSin3a Expression (%Sham)

B

WT  PI3Kγ-/-  PI3Kδ D910A/D910A

Sham
Smoke (S)
S+Bud

Nuclear Mi-2α Expression (%Sham)

C

WT  PI3Kγ-/-  PI3Kδ D910A/D910A

Sham
Smoke (S)
S+Bud

Nuclear Mi-2β Expression (%Sham)

Figure 7

A

WT  PI3Kγ-/-  PI3Kδ D910A/D910A

Sham
Smoke (S)
S+Bud

Cytosolic GR Expression (%Sham)

B

WT  PI3Kγ-/-  PI3Kδ D910A/D910A

Sham
Smoke (S)
S+Bud

Nuclear GR Expression (%Sham)
Figure 8

(A) Normal Smoker

(B) Normal Smoker COPD

(C) COPD

(D) Normal Smoker

(E) Normal Smoker COPD

(F) COPD

(G) GRα Expression (normalised to Actin)

(H) GRβ Expression (normalised to Actin)
Figure 1. Impact of oxidative stress on PI3K phosphorylation and glucocorticoid function in vitro. (A) Blocked induction of 200μM H₂O₂ Akt phosphorylation by 1μM LY294002. (B) 200μM H₂O₂ mediated reduction in dexamethasone inhibition of TNFα induced IL-8 release. (C) Restoration of dexamethasone inhibition of TNFα induced IL-8 release by 1μM LY294002. Abbreviations; LY: LY294002; Dex: Dexamethasone.

Figure 2. PI3KδD910A/D910A but not PI3Kγ⁻/⁻ restored budesonide mediated suppression of lung neutrophil recruitment in smoke exposed mice. All data represents the mean ± S.E.M (n=7-8). Abbreviations; S: Smoke Exposed; Bud: Budesonide.

Figure 3. Inflammatory cytokine profile of smoke exposed mouse lung; impact of PI3Kγ⁻/⁻ and PI3KδD910A/D910A. (A) Restored budesonide mediated reduction in lung tissue KC levels in PI3KδD910A/D910A but not PI3Kγ⁻/⁻ smoke exposed mice. (B) Restored budesonide mediated reduction in lung tissue IL-6 levels in PI3KδD910A/D910A but not PI3Kγ⁻/⁻ smoke exposed mice. All data represents the mean ± S.E.M (n=7-8). Abbreviations; S: Smoke Exposed; Bud: Budesonide.

Figure 4. HDAC-2 activity in smoke exposed mouse lung; impact of PI3Kγ⁻/⁻ and PI3KδD910A/D910A. (A) Restored total nuclear HDAC activity in PI3KδD910A/D910A but not PI3Kγ⁻/⁻ smoke exposed mice lungs. (B) Restored nuclear HDAC-2 activity in PI3KδD910A/D910A but not PI3Kγ⁻/⁻ smoke exposed mice lungs. All data represents the mean ± S.E.M (n=7-8). Abbreviations; S: Smoke Exposed; Bud: Budesonide.

Figure 5. HDAC-2 posttranslational modifications in smoke exposed mouse lung; impact of PI3Kγ⁻/⁻ and PI3KδD910A/D910A. (A) Reduced nuclear HDAC-2 tyrosine nitration in PI3KδD910A/D910A but not PI3Kγ⁻/⁻ smoke exposed mice lungs. (B) Reduced nuclear HDAC-2 serine phosphorylation in PI3KδD910A/D910A but not PI3Kγ⁻/⁻ smoke exposed mice lungs. Immunoblot is a representative image from n=7-8. Histograms represent the mean ± S.E.M (n=7-8). Abbreviations; S: Smoke Exposed; Bud: Budesonide; N-Tyr: Tyrosine Nitration; P-Ser: Serine Phosphorylation.
**Figure 6.** Expression of co-repressor complex proteins mSin3a and Mi-2 in smoke exposed mouse lung; impact of PI3Kγ−/− and PI3KδD910A/D910A. (A) Reduced nuclear mSin3a expression in smoke exposed mice lungs with no impact of either PI3Kγ−/− or PI3KδD910A/D910A. (B) Restored Mi-2α expression in both PI3Kγ−/− and PI3KδD910A/D910A smoke exposed mice lungs. (C) Elevated Mi-2β expression in smoke exposed mice lungs with no impact of either PI3Kγ−/− or PI3KδD910A/D910A. Immunoblot is a representative image from n=7-8. Histograms represent the mean ± S.E.M (n=7-8). **Abbreviations; S:** Smoke Exposed; **Bud:** Budesonide.

**Figure 7.** GRα expression in smoke exposed mouse lung; impact of PI3Kγ−/− and PI3KδD910A/D910A. (A) Reduced cytosolic GRα expression in smoke exposed mice lungs with no impact of either PI3Kγ−/− or PI3KδD910A/D910A. (B) Reduced nuclear GRα expression in smoke exposed mice lungs with no impact of either PI3Kγ−/−, PI3KδD910A/D910A or budesonide treatment. Immunoblot is a representative image from n=7-8. Histograms represent the mean ± S.E.M (n=7-8). **Abbreviations; S:** Smoke Exposed; **Bud:** Budesonide.

**Figure 8.** GR expression in the peripheral lung parenchyma of COPD lungs versus non-smoked and smokers. (A-F) GRα staining of the bronchiolar and alveolar epithelial cells, bronchiolar smooth muscle cells, endothelial cells and infiltrating cells. (G) Expression of GRα protein in peripheral lung of COPD patients (n=8) and smokers with normal lung function (n=9) compared to age-matched normal subjects (n=6). (H) Expression of GRβ protein in peripheral lung of COPD patients (n=8) and smokers with normal lung function (n=9) compared to age-matched normal subjects (n=6). Immunohistochemical and immunoblot pictures are representative images from n=6-9. Histograms represent the mean ± S.E.M (n=6-9).