Inhibition of PI3K δ 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 (GR α) 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 PI3K δ but not γ inhibition. GR α 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, PI3K γ knock-out and PI3K δ kinase dead knock-in transgenic mice were used in a model of cigarette smoke induced glucocorticoid insensitivity. Peripheral lung tissue was obtained from 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 PI3K δ kinase dead knock-in but not PI3K γ 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 PI3K δ may restore glucocorticoid function in oxidative stress induced glucocorticoid insensitivity.

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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; GR α , through which the actions of glucocorticoids are mediated, and the non glucocorticoid binding GR β (3). The major glucocorticoid anti-inflammatory action is mediated by transrepression of pro-inflammatory genes (4). Here, GR α monomers associate with promoter bound transcription factors such as nuclear factor NF- κ B and AP-1 (5) followed by recruitment 'co-repressor complexes' such as mSin3a (mammalian Sin3a) and Mi-2 α/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 GR α itself, thereby allowing NF- κ B binding and subsequent glucocorticoid-mediated transrepression of NF- κ B 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 PI3K δ and γ are proposed as potential anti-inflammatory targets (19;20) with PI3K δ implicated in B- and T- cell signalling, mast cell mediated allergic response and neutrophil activation and PI3K γ 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 PI3K δ but not γ inhibition with further alterations in lung mSin3a and Mi-2 α/β expression and a reduction in lung GR α expression. Furthermore, both GR α and GR β 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), budesonide (Sigma) and TNF α (R&D Systems, Abingdon, UK). *Antibodies:* Akt (New England BioLabs, Herts, UK); HDAC-2, GR α , lamin A/C, actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mSin3a, GAPDH, Nitrotyrosine and

phosphoserine (Abcam, Cambridge, UK); GR β (Affinity Bioreagents, CO, USA); Mi-2 α/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 PI3K $\gamma^{-/-}$ and PI3K $\delta^{D910A/D910A}$ 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 FEV₁/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 $\pm$ SEM, differences were considered significant if $P < 0.05$.

Results

Oxidative stress induces Akt phosphorylation and reduced glucocorticoid function in vitro. Hydrogen peroxide (H₂O₂) induced Akt phosphorylation in a time and PI3K-dependant manner (Fig. 1A). H₂O₂ 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 α (Fig. 1B). Pre-treatment with 100nM dexamethasone gave a maximal inhibition of TNF α mediated IL-8 release (~50-60%), however in cells exposed to H₂O₂, 100nM dexamethasone was only able to reduced TNF α induced IL-8 levels to that comparable to TNF α alone (Fig. 1B). Inhibition of PI3K restored dexamethasone suppression of IL-8 release with no apparent impact alone (Fig. 1C).

Inhibition of PI3K δ 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 PI3K δ kinase dead knock-in PI3K $\delta^{D910A/D910A}$ or PI-3K γ knockout PI3K $\gamma^{-/-}$ 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 PI3K $\delta^{D910A/D910A}$ mice, but not PI3K $\gamma^{-/-}$ reduced both the neutrophil influx and lung tissue cytokine levels, indicating that the PI3K δ pathway may play a role in the development of cigarette smoke induced glucocorticoid insensitivity.

Inhibition of PI3K δ 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 PI3K $\gamma^{-/-}$ mice lungs (Fig. 4 A, B). However, in smoke exposed PI3K $\delta^{D910A/D910A}$ 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 PI3K $\gamma^{-/-}$ (Fig. 5A, B). However, both tyrosine nitration and serine phosphorylation of HDAC-2 were reduced in smoke exposed PI3K $\delta^{D910A/D910A}$ mice compared to WT controls (Fig. 5 A, B). Budesonide treatment had no additional impact on either tyrosine nitration or serine phosphorylation in any group.

Cigarette smoke exposure alters mSin3a and Mi-2 α/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-2 α/β which co-ordinate and orchestrate the HDAC-2 co-repressor complex. Both mSin3a and Mi-2 α expression was reduced in cigarette smoke exposed mice with no impact of either PI3K δ or γ inhibition (Fig. 6A, B). Interestingly, budesonide treatment prevented the reduction of mSin3a expression in all groups (Fig. 6A), however Mi-2 α expression was only maintained in the budesonide treated PI3K $\gamma^{-/-}$, PI3K $\delta^{D910A/D910A}$ mice (Fig. 6B). Conversely, Mi-2 β expression was elevated in all smoke exposed groups with no discernable difference between PI3K δ 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 GR α itself. There was no difference in GR α protein expression between WT and PI3K $\gamma^{-/-}$ or PI3K $\delta^{D910A/D910A}$ sham mice in either the cytosolic or nuclear compartments. Cigarette smoke exposure significantly reduced GR α

protein expression with no impact of either PI3K δ or γ inhibition (Fig. 7 A, B). However, there was also no difference in the GR α cytosolic:nuclear ratios in the smoke exposed mice with and without budesonide treatment (data not shown) indicating that budesonide mediated GR α translocation is ineffective in smoke exposed animals. To assess if this reduction in GR α 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 GR α 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 GR α and GR β 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 PI3K δ 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-2 α/β . Although oxidative stress reduced GR α expression, restoration of GR α function in PI3K $\delta^{D910/D910}$ 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 $PI3K\gamma^{-/-}$ and $PI3K\delta^{D910A/D910A}$ mice indicating that acutely, neither of these isoforms impact on oxidant induced inflammation. Contrary to this, both pharmacological and knock out studies show that $PI3K\gamma$ 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 $PI3K\gamma^{-/-}$ or $PI3K\delta^{D910A/D910A}$ to be seen (34). Interestingly, budesonide pre-treatment had no impact on the inflammatory response in $PI3K\gamma^{-/-}$ mice, but reduced the inflammatory response in $PI3K\delta^{D910A/D910A}$ mice indicating that oxidative induced glucocorticoid insensitivity may be linked to activation of $PI3K\delta$ specific signalling. However the specific signalling pathways of the $PI3K\delta$ 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 $GR\alpha$ levels in the nuclear compartment with budesonide, indicating that cigarette smoke exposure may impact on $GR\alpha$ translocation. However, as glucocorticoid function was restored in $PI3K\delta^{D910A/D910A}$ mice with no further impact on $GR\alpha$ expression or translocation, the endogenous levels of nuclear $GR\alpha$ may have been sufficient to provide relative restoration of the anti-inflammatory transrepression without elevated $GR\alpha$ translocation. This data therefore suggests that the reduction of $GR\alpha$ 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 $GR\alpha$

expression is reduced in the periphery of the lung of smokers and is further reduced in the lungs from patients with the glucocorticoid-insensitive disease COPD. Furthermore, lung GR β expression was also reduced in both smokers and COPD, indicating that GR β is unlikely to play a significant role in the development of glucocorticoid insensitivity in COPD.

HDAC-2 activity is critical to GR α 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 PI3K δ but not PI3K γ protected nuclear total HDAC and HDAC-2 activity, correlating with the glucocorticoid insensitivity in smoke exposed wt and PI3K $\gamma^{-/-}$ but not PI3K $\delta^{D910A/D910A}$ 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 PI3K $\delta^{D910A/D910A}$ 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 GR α /HDAC-2 co-repressor complexes. The co-repressor 'scaffold' proteins mSin3a and Mi-2 α/β are thought to coordinate the construction of the co-repressor complexes to deliver both HDAC and methyl transferase 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-2 α expression in the lung whilst Mi-2 β was conversely elevated. Interestingly, glucocorticoid treatment elevated mSin3a expression independent of PI3K γ/δ inhibition, whilst only restoring Mi-2 α expression in mice with PI-3K γ/δ inhibition. To our knowledge this is the first time that the direct impact oxidative stress on the expression of either mSin3a or Mi-2 α/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 PI3K δ by a mechanism that involves the restoration of HDAC-2 activity. Furthermore, oxidant induced reduction of GR α expression, impaired translocation and alteration in mSin3a and M2 α may limiting the level of nuclear GR α available for transrepression, thereby representing represent additional mechanisms by which oxidative stress impairs glucocorticoid sensitivity. Clinically, the development of PI3K δ 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

1. Barnes,P.J. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008; **8**:183-192.
2. Chikanza,I.C., and Kozaci,D.L. Corticosteroid resistance in rheumatoid arthritis: molecular and cellular perspectives. *Rheumatology* 2004; **43**:1337-1345.
3. Goleva,E., Li,L.b., Eves,P.T., Strand,M.J., Martin,R.J., and Leung,D.Y.M. Increased Glucocorticoid Receptor beta Alters Steroid Response in Glucocorticoid-insensitive Asthma. *Am J Respir Crit Care Med* 2006; **173**:607-616.
4. Reichardt,H.M., Kaestner,K.H., Tuckermann,J., Kretz,O., Wessely,O., Bock,R., Gass,P., Schmid,W., Herrlich,P., Angel,P. et al DNA Binding of the Glucocorticoid Receptor Is Not Essential for Survival. *Cell* 1998; **93**:531-541.
5. Glass,C.K., and Ogawa,S. Combinatorial roles of nuclear receptors in inflammation and immunity. *Nat Rev Immunol* 2006; **6**:44-55.
6. Hassig,C.A., Fleischer,T.C., Billin,A.N., Schreiber,S.L., and Ayer,D.E. Histone Deacetylase Activity Is Required for Full Transcriptional Repression by mSin3A. *Cell* 1997; **89**:341-347.
7. Knoepfler,P.S., and Eisenman,R.N. Sin Meets NuRD and Other Tails of Repression. *Cell* 1999; **99**:447-450.
8. Tyler,J.K., and Kadonaga,J.T. The "Dark Side" of Chromatin Remodeling: Repressive Effects on Transcription. *Cell* 1999; **99**:443-446.

9. Ito,K., Barnes,P.J., and Adcock,I.M. Glucocorticoid Receptor Recruitment of Histone Deacetylase 2 Inhibits Interleukin-1beta -Induced Histone H4 Acetylation on Lysines 8 and 12. *Mol Cell Biol* 2000; **20**:6891-6903.
10. Ito,K., Yamamura,S., Essilfie-Quaye,S., Cosio,B., Ito,M., Barnes,P.J., and Adcock,I.M. Histone deacetylase 2-mediated deacetylation of the glucocorticoid receptor enables NF- κ B suppression. *J Exp Med* 2006; **203**:7-13.
11. Li,B., Carey,M., and Workman,J.L. The Role of Chromatin during Transcription. *Cell* 2007; **128**:707-719.
12. Ito,K., Ito,M., Elliott,W.M., Cosio,B., Caramori,G., Kon,O.M., Barczyk,A., Hayashi,S., Adcock,I.M., Hogg,J.C. et al. Decreased Histone Deacetylase Activity in Chronic Obstructive Pulmonary Disease. *N Engl J Med* 2005; **352**:1967-1976.
13. Hew,M., Bhavsar,P., Torrego,A., Meah,S., Khorasani,N., Barnes,P.J., Adcock,I., Fan Chung,K., and for the National Heart Lung and Blood Institute's Severe Asthma Research Program. Relative Corticosteroid Insensitivity of Peripheral Blood Mononuclear Cells in Severe Asthma. *Am J Respir Crit Care Med* 2006; **174**:134-141.
14. Ito,K., Lim,S., Caramori,G., Chung,K.F., Barnes,P.J., and Adcock,I.M. Cigarette smoking reduces histone deacetylase 2 expression, enhances cytokine expression, and inhibits glucocorticoid actions in alveolar macrophages. *FASEB J.* 2001; **15**:1110-1112.
15. Ukena,D., Harnest,U., Sakalauskas,R., Magyar,P., Vetter,N., Steffen,H., Leichtl,S., Rathgeb,F., Keller,A., and Steinijans,V.W. Comparison of addition of theophylline to inhaled steroid with doubling of the dose of inhaled steroid in asthma. *Eur Respir J* 1997; **10**:2754-2760.

16. Cosio,B.G., Tsaprouni,L., Ito,K., Jazrawi,E., Adcock,I.M., and Barnes,P.J. Theophylline restores histone deacetylase activity and steroid responses in COPD macrophages. *J Exp Med* 2004; **200**:689-695.
17. Ito,K., Lim,S., Caramori,G., Cosio,B., Chung,K.F., Adcock,I.M., and Barnes,P.J. A molecular mechanism of action of theophylline: Induction of histone deacetylase activity to decrease inflammatory gene expression. *Proc. Natl. Acad. Sci.* 2002; **99**:8921-8926.
18. Foukas,L.C., Daniele,N., Ktori,C., Anderson,K.E., Jensen,J., and Shepherd,P.R. Direct Effects of Caffeine and Theophylline on p110 δ and Other Phosphoinositide 3-Kinases; Differential effects on lipid kinase activities. *J. Biol. Chem.* 2002; **277**:37124-37130.
19. Condliffe,A.M., Davidson,K., Anderson,K.E., Ellson,C.D., Crabbe,T., Okkenhaug,K., Vanhaesebroeck,B., Turner,M., Webb,L., Wymann,M.P. et al. Sequential activation of class IB and class IA PI3K is important for the primed respiratory burst of human but not murine neutrophils. *Blood* 2005; **106**:1432-1440.
20. Rommel,C., Camps,M., and Ji,H. PI3K δ and PI3K γ : partners in crime in inflammation in rheumatoid arthritis and beyond? *Nat Rev Immunol* 2007; **7**:191-201.
21. Sasaki,T., Irie-Sasaki,J., Jones,R.G., Oliveira-dos-Santos,A.J., Stanford,W.L., Bolon,B., Wakeham,A., Itie,A., Bouchard,D., Kozieradzki,I. et al. Function of PI3K in Thymocyte Development, T Cell Activation, and Neutrophil Migration. *Science* 2000; **287**:1040-1046.
22. Puri,K.D., Doggett,T.A., Douangpanya,J., Hou,Y., Tino,W.T., Wilson,T., Graf,T., Clayton,E., Turner,M., Hayflick,J.S. et al. Mechanisms and implications of

- phosphoinositide 3-kinase δ in promoting neutrophil trafficking into inflamed tissue. *Blood* 2004; **103**:3448-3456.
23. Ali,K., Bilancio,A., Thomas,M., Pearce,W., Gilfillan,A.M., Tkaczyk,C., Kuehn,N., Gray,A., Giddings,J., Peskett,E. et al. Essential role for the p110 δ phosphoinositide 3-kinase in the allergic response. *Nature* 2004; **431**:1007-1011.
 24. Lee,K.S., Lee,H.K., Hayflick,J.S., Lee,Y.C., and Puri,K.D. Inhibition of phosphoinositide 3-kinase δ attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *FASEB J.* 2006; **20**:455-465.
 25. Hirsch,E., Katanaev,V.L., Garlanda,C., Azzolino,O., Pirola,L., Silengo,L., Sozzani,S., Mantovani,A., Altruda,F., and Wymann,M.P. Central Role for G Protein-Coupled Phosphoinositide 3-Kinase gamma in Inflammation. *Science* 2000; **287**:1049-1053.
 26. Okkenhaug,K., Bilancio,A., Farjot,G., Priddle,H., Sancho,S., Peskett,E., Pearce,W., Meek,S.E., Salpekar,A., Waterfield,M.D. et al. Impaired B and T Cell Antigen Receptor Signaling in p110delta PI 3-Kinase Mutant Mice. *Science* 2002; **297**:1031-1034.
 27. Stevenson,C.S., Coote,K., Webster,R., Johnston,H., Atherton,H.C., Nicholls,A., Giddings,J., Sugar,R., Jackson,A., Press,N.J. et al. Characterization of cigarette smoke-induced inflammatory and mucus hypersecretory changes in rat lung and the role of CXCR2 ligands in mediating this effect. *Am J Physiol Lung Cell Mol Physiol* 2005; **288**:L514-L522.
 28. Marwick,J.A., Kirkham,P.A., Stevenson,C.S., Danahay,H., Giddings,J., Butler,K., Donaldson,K., MacNee,W., and Rahman,I. Cigarette Smoke Alters Chromatin Remodeling and Induces Proinflammatory Genes in Rat Lungs. *Am J Respir Cell Mol Biol* 2004; **31**:633-642.

29. Varani,K., Caramori,G., Vincenzi,F., Adcock,I., Casolari,P., Leung,E., MacLennan,S., Gessi,S., Morello,S., Barnes,P.J. et al. Alteration of Adenosine Receptors in Patients with Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 2006; **173**:398-406.
30. Rabe,K.F., Hurd,S., Anzueto,A., Barnes,P.J., Buist,S.A., Calverley,P., Fukuchi,Y., Jenkins,C., Rodriguez-Roisin,R., van Weel,C. et al. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease: GOLD Executive Summary. *Am J Respir Crit Care Med* 2007; **176**:532-555.
31. Sadhu,C., Masinovsky,B., Dick,K., Sowell,C.G., and Staunton,D.E. Essential Role of Phosphoinositide 3-Kinase δ in Neutrophil Directional Movement. *J Immunol* 2003; **170**:2647-2654.
32. Yang,K.Y., Arcaroli,J., Kupfner,J., Pitts,T.M., Park,J.S., Strasshiem,D., Perng,R.P., and Abraham,E. Involvement of phosphatidylinositol 3-kinase γ in neutrophil apoptosis. *Cellular Signalling* 2003; **15**:225-233.
33. Pinho,V., Souza,D.G., Barsante,M.M., Hamer,F.P., De Freitas,M.S., Rossi,A.G., and Teixeira,M.M. Phosphoinositide-3 kinases critically regulate the recruitment and survival of eosinophils in vivo: importance for the resolution of allergic inflammation. *J Leukoc Biol* 2005; **77**:800-810.
34. Grummelli,S.M., Lu,B., Shapiro,S.D., and Gerard,C. Decreased Inflammation in the Smoking Model of PI3K Knock-Out Mice. *Am J Respir Crit Care Med* 2007; **175**:A684 (Abstr.)

35. Galasinski,S.C., Resing,K.A., Goodrich,J.A., and Ahn,N.G. Phosphatase Inhibition Leads to Histone Deacetylases 1 and 2 Phosphorylation and Disruption of Corepressor Interactions. *J Biol Chem* 2002; **277**:19618-19626.
36. Ito,K., Hanazawa,T., Tomita,K., Barnes,P.J., and Adcock,I.M. Oxidative stress reduces histone deacetylase 2 activity and enhances IL-8 gene expression: role of tyrosine nitration. *Biochem Biophys Res Commun* 2004; **315**:240-245.

Table 1: Characteristics of subjects for the study

Subjects	n	Age	Sex	Smoking history	Pack-years	Chronic bronchitis	FEV ₁ [*] % pred	FEV ₁ /FVC [†] %
NS [‡]	6	70.0±3.0	4M [§] ,/2F ^{**}	None	0	None	102.2±5.6	77.7±3.1
Smoker	9	67.3±2.6	8M/1F	5 Ex ^{††} : 4 Cur ^{‡‡}	30.1±5.5	6 yes : 3 no	95.9±6.1	76.0±1.3
COPD	8	70.0±1.6	8M	4 Ex : 4 Cur	39±5.2	6 yes : 2 no	85.3±3.7	66.5±1.0

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 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 1. Impact of oxidative stress on PI3K phosphorylation and glucocorticoid function *in vitro*. (A) Blocked induction of 200 μ M H₂O₂ Akt phosphorylation by 1 μ M LY294002. (B) 200 μ M H₂O₂ mediated reduction in dexamethasone inhibition of TNF α induced IL-8 release. (C) Restoration of dexamethasone's inhibition of TNF α induced IL-8 release by 1 μ M LY294002. *Abbreviations; LY: LY294002; Dex: Dexamethasone.*

Figure 2. PI3K $\delta^{D910A/D910A}$ but not PI3K $\gamma^{-/-}$ restored budesonide mediated suppression of lung neutrophil recruitment in smoke exposed mice. All data represents the mean $\pm$ S.E.M (n=7-8). *Abbreviations; S: Smoke Exposed; Bud: Budesonide.*

Figure 3. Inflammatory cytokine profile of smoke exposed mouse lung; impact of PI3K $\gamma^{-/-}$ and PI3K $\delta^{D910A/D910A}$. (A) Restored budesonide mediated reduction in lung tissue KC levels in PI3K $\delta^{D910A/D910A}$ but not PI3K $\gamma^{-/-}$ smoke exposed mice. (B) Restored budesonide mediated reduction in lung tissue IL-6 levels in PI3K $\delta^{D910A/D910A}$ but not PI3K $\gamma^{-/-}$ smoke exposed mice. All data represents the mean $\pm$ S.E.M (n=7-8). *Abbreviations; S: Smoke Exposed; Bud: Budesonide.*

Figure 4. HDAC-2 activity in smoke exposed mouse lung; impact of PI3K $\gamma^{-/-}$ and PI3K $\delta^{D910A/D910A}$. (A) Restored total nuclear HDAC activity in PI3K $\delta^{D910A/D910A}$ but not PI3K $\gamma^{-/-}$ smoke exposed mice lungs. (B) Restored nuclear HDAC-2 activity in PI3K $\delta^{D910A/D910A}$ but not PI3K $\gamma^{-/-}$ smoke exposed mice lungs. All data represents the mean $\pm$ 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 PI3K $\gamma^{-/-}$ and PI3K $\delta^{D910A/D910A}$. (A) Reduced nuclear HDAC-2 tyrosine nitration in PI3K $\delta^{D910A/D910A}$ but not PI3K $\gamma^{-/-}$ smoke exposed mice lungs. (B) Reduced nuclear HDAC-2 serine phosphorylation in PI3K $\delta^{D910A/D910A}$ but not PI3K $\gamma^{-/-}$ smoke exposed mice lungs. Immunoblot is a representative image from n=7-8. Histograms represent the mean $\pm$ 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 PI3K $\gamma^{-/-}$ and PI3K $\delta^{D910A/D910A}$. (A) Reduced nuclear mSin3a expression in smoke exposed mice lungs with no impact of either PI3K $\gamma^{-/-}$ or PI3K $\delta^{D910A/D910A}$. (B) Restored Mi-2 α expression in both PI3K $\gamma^{-/-}$ and PI3K $\delta^{D910A/D910A}$ smoke exposed mice lungs. (C) Elevated Mi-2 β expression in smoke exposed mice lungs with no impact of either PI3K $\gamma^{-/-}$ or PI3K $\delta^{D910A/D910A}$. Immunoblot is a representative image from n=7-8. Histograms represent the mean $\pm$ S.E.M (n=7-8). *Abbreviations; S: Smoke Exposed; Bud: Budesonide.*

Figure 7. GR α expression in smoke exposed mouse lung; impact of PI3K $\gamma^{-/-}$ and PI3K $\delta^{D910A/D910A}$. (A) Reduced cytosolic GR α expression in smoke exposed mice lungs with no impact of either PI3K $\gamma^{-/-}$ or PI3K $\delta^{D910A/D910A}$. (B) Reduced nuclear GR α expression in smoke exposed mice lungs with no impact of either PI3K $\gamma^{-/-}$, PI3K $\delta^{D910A/D910A}$ or budesonide treatment. Immunoblot is a representative image from n=7-8. Histograms represent the mean $\pm$ 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) GR α staining of the bronchiolar and alveolar epithelial cells, bronchiolar smooth muscle cells, endothelial cells and infiltrating cells. (G) Expression of GR α 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 GR β 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 $\pm$ S.E.M (n=6-9).