

## RESEARCH PAPER

# Corticosteroid-induced gene expression in allergen-challenged asthmatic subjects taking inhaled budesonide

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## BACKGROUND AND PURPOSE

Inhaled corticosteroids (ICS) are the cornerstone of asthma pharmacotherapy and, acting via the glucocorticoid receptor (GR), reduce inflammatory gene expression. While this is often attributed to a direct inhibitory effect of the GR on inflammatory gene transcription, corticosteroids also induce the expression of anti-inflammatory genes *in vitro*. As there are no data to support this effect in asthmatic subjects taking ICS, we have assessed whether ICS induce anti-inflammatory gene expression in subjects with atopic asthma.

## EXPERIMENTAL APPROACH

Bronchial biopsies from allergen-challenged atopic asthmatic subjects taking inhaled budesonide or placebo were subjected to gene expression analysis using real-time reverse transcriptase-PCR for the corticosteroid-inducible genes (official gene symbols with aliases in parentheses): TSC22D3 [glucocorticoid-induced leucine zipper (GILZ)], dual-specificity phosphatase-1 (MAPK phosphatase-1), both anti-inflammatory effectors, and FKBP5 [FK506-binding protein 51 (FKBP51)], a regulator of GR function. Cultured pulmonary epithelial and smooth muscle cells were also treated with corticosteroids before gene expression analysis.

## KEY RESULTS

Compared with placebo, GILZ and FKBP51 mRNA expression was significantly elevated in budesonide-treated subjects. Budesonide also increased GILZ expression in human epithelial and smooth muscle cells in culture. Immunostaining of bronchial biopsies revealed GILZ expression in the airways epithelium and smooth muscle of asthmatic subjects.

## CONCLUSIONS AND IMPLICATIONS

Expression of the corticosteroid-induced genes, GILZ and FKBP51, is up-regulated in the airways of allergen-challenged asthmatic subjects taking inhaled budesonide. Consequently, the biological effects of corticosteroid-induced genes should be considered when assessing the actions of ICS. Treatment modalities that increase or decrease GR-dependent transcription may correspondingly affect corticosteroid efficacy.

## Abbreviations

AP-1, activator protein 1; ASM, airway smooth muscle; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FKBP51, FK506-binding protein 51; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GILZ, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HBE, human bronchial epithelial; ICS, inhaled corticosteroid; MKP, MAPK phosphatase; RT, reverse transcriptase

## Introduction

In asthma, inhaled corticosteroids (ICS), also called glucocorticoids, are highly effective at reducing inflammation and improving patient outcomes (Barnes, 2006), and ICS remain the cornerstone of pharmacological therapy (Bateman *et al.*, 2008). ICS attenuate airway eosinophil numbers, reduce asthma exacerbations and mortality and lead to improvements in lung function and health-related quality of life (Suisa *et al.*, 2000; Pauwels *et al.*, 2003; Kelly *et al.*, 2006b; Busse *et al.*, 2008; Newton *et al.*, 2010b). However, in many individuals, asthma remains poorly controlled, even when using high-dose ICS or systemic corticosteroids (Cazzoletti *et al.*, 2007; Peters *et al.*, 2007). Furthermore, corticosteroids may be less effective at controlling neutrophilic or non-eosinophilic inflammation, which often predominates in viral exacerbations of asthma and in cigarette smokers. This latter observation is likely to account for the relative insensitivity of chronic obstructive pulmonary disease to ICS therapy (Adcock and Barnes, 2008; Newton *et al.*, 2010b).

At the molecular level, corticosteroids repress expression of inflammatory genes including cytokines, chemokines, adhesion molecules and others, and this effect is central to their anti-inflammatory action (Barnes, 2006). Mechanistically, corticosteroids bind the glucocorticoid receptor (GR), which then translocates from the cytoplasm into the nucleus. The prevailing paradigm holds that GR directly prevents inflammatory gene transcription by reducing transcriptional activation via key inflammatory factors such as NF- $\kappa$ B and activator protein 1 (AP-1) (Barnes, 2006). This mechanism, called transrepression, forms the mainstay of our perception as to how corticosteroids repress inflammatory gene expression. However, transrepression cannot readily account for observations that transcriptional and/or translational inhibitors prevent the repressive effects of corticosteroids on inflammatory gene expression (for examples, see: Ristimäki *et al.*, 1996; Newton *et al.*, 1998, 2010a; Lasa *et al.*, 2001; Chivers *et al.*, 2006). Rather, such data point to indirect actions by inducing expression of anti-inflammatory genes, which then repress inflammatory gene expression (Stellato, 2004; Clark, 2007; Newton and Holden, 2007). Indeed, many hundreds of genes are induced by corticosteroids and many of these may exert anti-inflammatory effects (Newton and Holden, 2007). For example, MAPK phosphatase (MKP) 1 (dual-specificity phosphatase-1) is rapidly and highly induced by corticosteroids and inhibits MAPK pathways to cause mRNA destabilization, repression of transcription via factors such as NF- $\kappa$ B and AP-1, as well as probably reducing inflammatory gene translation (Kassel *et al.*, 2001; Lasa *et al.*, 2002; Clark *et al.*, 2008; King *et al.*, 2009; Newton *et al.*, 2010a). Similarly, glucocorticoid-induced leucine zipper (GILZ) (TSC22D3) is profoundly induced by corticosteroids and represses NF- $\kappa$ B-dependent transcription and other inflammatory signalling pathways leading to AP-1 activation (Mittelstadt and Ashwell, 2001; Ayroldi *et al.*, 2007; Eddleston *et al.*, 2007). Alternatively, increased expression of genes such as FK506-binding protein 51 (FKBP51), which is believed to negatively regulate GR function (Davies *et al.*, 2002), may exert a dampening effect on glucocorticoid responses and could thereby reduce anti-inflammatory effects (Stechschulte and Sanchez, 2011).

However, despite the increasing body of evidence supporting key anti-inflammatory or regulatory effects of corticosteroid-induced genes, there is no direct evidence that such effects occur in asthmatic patients taking ICS. To address this fundamental issue, the current study has examined bronchial biopsies from the airways of allergen-challenged patients with mild atopic asthma taking inhaled budesonide to assess corticosteroid-inducible gene expression. These responses are compared with the effects of dexamethasone and budesonide in human epithelial and smooth muscle cells *in vitro*.

## Methods

### Cell culture and drugs

Human A549 pulmonary epithelial and human bronchial airway epithelial BEAS-2B cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) or DMEM/F12 (both Invitrogen, Burlington, Ontario, Canada), respectively, supplemented with 10% fetal calf serum (FCS) (Invitrogen) as described previously (King *et al.*, 2009). Primary human bronchial epithelial (HBE) cells were prepared from normal human lung and were cultured in bronchial epithelial cell growth medium (BioWhittaker, Walkersville, MD, USA) (Proud *et al.*, 2004). Human airways smooth muscle (ASM) cells were prepared from smooth muscle bundles obtained from normal human lung and grown in DMEM supplemented with 10% FCS as previously described (Kaur *et al.*, 2008a). On reaching confluence, cells were maintained in serum- or supplement-free media overnight before the drug treatments. Dexamethasone and budesonide were from Sigma (Oakville, Ontario, Canada), and human recombinant IL-1 $\beta$  was from R&D Systems (Hornby, Ontario, Canada).

### Biopsy samples and RNA extraction

Biopsy samples were derived from a previously described cross-over study (Kelly *et al.*, 2010). Briefly, in this allergen-challenged model, patients with mild atopic asthma were initially subjected to a 3 week washout period before taking an inhaled diluent (saline). The following day, they underwent a baseline bronchoscopy where mucosal biopsies were obtained. Thereafter, patients were randomly allocated to the three treatment groups (inhaled placebo, budesonide or budesonide plus formoterol) of the original study. Each volunteer received all treatments in a sequential, randomized, cross-over study design. Biopsy samples obtained from the initial saline challenge and from the placebo and budesonide arms were used for analysis in the current investigation. Before each treatment period, there was a 3 week washout. Patients then received inhaled placebo or study drug for 11 consecutive days. Budesonide was inhaled using a Pulmicort Turbuhaler (budesonide, 200  $\mu$ g) with two inhalations taken twice daily (total daily dose 800  $\mu$ g). Placebo Turbuhaler was administered in a similar manner. On day 9 of each treatment period, subjects underwent an inhaled allergen challenge using an amount of allergen that was two doubling concentrations less than that predicted to cause a 20% decrease in forced expiratory volume in 1 second (FEV<sub>1</sub>) as described previously (Kelly *et al.*, 2010). On day 10, bronchoscopy was

performed to obtain post-treatment mucosal biopsies. These biopsies were immediately frozen on dry ice in Tissue-Tek OCT (Sakura Finetek, Torrance, CA, USA) and stored at  $-80^{\circ}\text{C}$  before total RNA extraction as described (Kelly *et al.*, 2010). Frozen biopsies were sectioned with a clean cryostat blade onto glass slides. Tissue was removed for RNA extraction using the PicoPure<sup>TM</sup> RNA isolation kit (Molecular Devices, Sunnyvale, CA, USA) and followed by DNase treatment (Qiagen, Mississauga, Ontario, Canada). RNA quality and concentration was assessed by Agilent 2100 bioanalyser (Agilent Technologies, Santa Clara, CA, USA). Biopsy RNA samples obtained from the initial diluent challenge and the placebo and budesonide arms were analysed for expression of corticosteroid-induced genes. RNA from cultured cells was extracted using RNeasy Mini Kits (Qiagen).

### Reverse transcription-PCR (RT-PCR)

RNA, 0.5  $\mu\text{g}$  from cells or 5 ng from biopsies, was used to generate cDNA using the qScript cDNA kit (Quanta Bio-Sciences, Inc, Gaithersburg, MD, USA) in a final volume of 20  $\mu\text{L}$ . The resultant cDNA was diluted fivefold and 2.5  $\mu\text{L}$  used in 20  $\mu\text{L}$  real-time PCR reactions using Sybr GreenER chemistry (Invitrogen) and an ABI 7900HT instrument (Applied Biosystems Inc, Foster City, CA, USA). Relative cDNA concentrations were obtained from a common cDNA standard curve. Amplification conditions were:  $50^{\circ}\text{C}$ , 2 min;  $95^{\circ}\text{C}$ , 10 min; then 40 cycles of  $95^{\circ}\text{C}$ , 15 s;  $60^{\circ}\text{C}$ , 1 min. Reactions were followed by a melt analysis ( $95^{\circ}\text{C}$  for 15 s,  $60^{\circ}\text{C}$  for 20 s,  $95^{\circ}\text{C}$  for 15 s with ramping to  $95^{\circ}\text{C}$  over 20 min) to confirm primer specificity. Amplification primers (5' – 3') were: glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (NM\_002046), forward (F) – TTC ACC ACC ATG GAG AAG GC, reverse (R) – AGG AGG CAT TGC TGA TGA TCT; GILZ (NM\_198057.2, NM\_004089.3, NM\_001015881.1), F – GGC CAT AGA CAA CAA GAT CG, R – ACT TAC ACC GCA GAA CCA CCA; FKBP51 (NM\_004117), F – CAG CTG CTC ATG AAC GAG TTT G, R – GCT TTA TTG GCC TCT TCC TTG G.

### Western blotting

Western blotting was as described previously (King *et al.*, 2009), except that lysates were size-fractionated on 12% SDS acrylamide gels. Following electrotransfer, Hybond-ECL membranes (GE Healthcare Bio-Sciences Inc, Baie d'Urfé, Quebec, Canada) were incubated with a mouse anti-sera for GAPDH (Cat no. 4699–9555, Biogenesis, AbD Serotec, Raleigh, NC, USA) or a previously described rabbit anti-serum for GILZ (Eddleston *et al.*, 2007). After being washed, membranes were incubated with horseradish peroxidase-linked secondary immunoglobulin (Dako, Mississauga, Ontario, Canada). Immune complexes were detected using enhanced chemiluminescence (GE Healthcare Bio-Sciences Inc) and visualized by autoradiography.

### Immunohistochemistry

Immunohistochemistry was performed on tissue sections using the streptavidin–biotin peroxidase method, according to the manufacturer's instructions (Vector Laboratories, Burlington, ON, USA), as previously described (Kelly *et al.*, 2006a). Briefly, after paraffin removal and rehydration, endogenous peroxidase activity was blocked with 3% hydro-

gen peroxide at room temperature for 20 min followed by successive treatment with avidin and biotin block (Vector Laboratories). GILZ antibody (100  $\mu\text{L}$ ) diluted 1/5000 in antibody diluent (Dako) or, as a negative control, rabbit isotype antibody (Dako) was applied to slides for 16–20 h at  $4^{\circ}\text{C}$ . After being washed, secondary biotinylated antibody (Vector goat anti-rabbit) at  $7.5 \mu\text{g}\cdot\text{mL}^{-1}$  was applied for 45 min, followed by Vector ABC avidin–biotin complex for 30 min at room temperature. Immunocomplexes were visualized with diaminobenzidine (Sigma Fast DAB, Sigma). Slides were counterstained with Surgipath Gills II haematoxylin (Leica Microsystems, Winnipeg, Canada) before dehydration through graded ethanols, clearing in xylene and mounting. Coded slides were analysed using a validated semi-quantitative scoring method based on the percentage of bronchial epithelium showing positive immunoreactive staining (Walker, 2006). Five categories were used where: 0 is no staining; 1, <25%; 2, 25–50%; 3, 50–75%; and 4, >75% staining.

### Statistical analysis

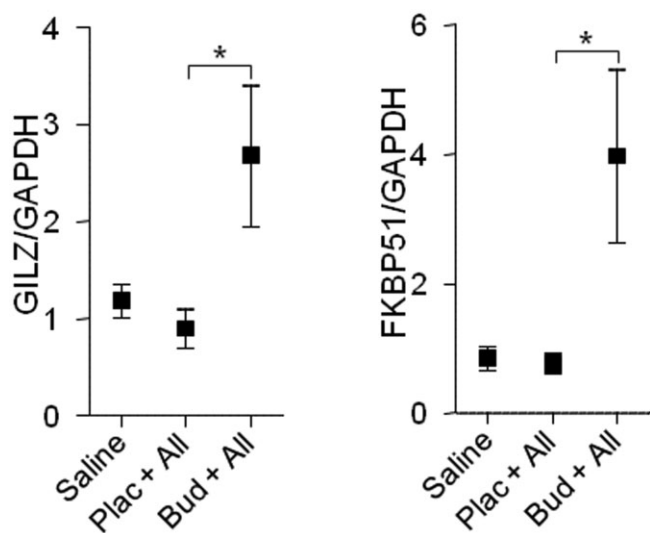
All graphical data are presented as mean  $\pm$  SEM. Statistical analysis between multiple groups was performed using non-parametric one-way ANOVA (Friedman's test) with a Dunn's post-test. Non-parametric *t*-tests were used to compare between two groups alone. Significance between groups was assumed where \**P* < 0.05, \*\**P* < 0.01 or \*\*\**P* < 0.001.

## Results

### Expression of corticosteroid-induced genes in the airways of asthmatic subjects taking inhaled budesonide

Prior microarray data identified GILZ and FKBP51 as the two most highly induced genes (18.9- and 10.8-fold, respectively) following 6 h treatment of pulmonary A549 epithelial cells with dexamethasone (Chivers *et al.*, 2006; Kaur *et al.*, 2008a). Similarly, MKP-1 was the most highly induced gene following 2 h of dexamethasone treatment. Furthermore, all three genes were consistent among the genes most highly up-regulated by dexamethasone at other times in A549 cells, and all show robust induction by corticosteroids in HBE BEAS-2B cells (Chivers *et al.*, 2006; Kaur *et al.*, 2008a). We therefore reasoned that these genes represent suitable targets for gene expression analysis in the bronchial biopsy samples. Following RNA extraction and cDNA generation, real-time PCR data, normalized to GAPDH, showed no significant change in the expression of GILZ, MKP-1 and FKBP51 between the initial saline challenge and the allergen-exposed placebo group (Figure 1, Supporting Information Figure S1A). Following budesonide treatment, GILZ and FKBP51 mRNA expression was significantly, 3.8- ( $\pm 1.4$ ) and 7.1-fold ( $\pm 3.6$ ) ( $\pm$ SEM), respectively, increased relative to placebo (Figure 1). A similar upward trend was observed in respect of MKP-1, but this did not reach significance (Supporting Information Figure S1A).

To examine the effect of budesonide treatment on the expression of inflammatory gene expression following allergen challenge, real-time RT-PCR was performed for IL-1 $\beta$ , CXCL8 and CCL5. Expression of IL-1 $\beta$  mRNA was at, or very



**Figure 1**

mRNA expression of corticosteroid-inducible genes in bronchial biopsies from mild atopic asthmatics taking inhaled budesonide. Mild asthmatics were entered into a previously described cross-over study (Kelly *et al.*, 2010). Patients were subjected to an initial 3 week washout period before an inhaled challenge with diluent alone (saline). Following this, patients were subjected to a further 3 week washout period before treatment with placebo (Plac) or drugs for 11 consecutive days in a randomized cross-over design. Budesonide (Bud) was inhaled using a Pulmicort Turbuhaler (budesonide, 200 µg) with two inhalations taken twice daily and placebo was administered in a similar manner. On day 9, patients were subjected to a mild allergen inhalation (All). Bronchoscopy was performed on day 10 (24 h after the allergen challenge). Biopsies were analysed for mRNA expression of GAPDH, GILZ and FKBP51 using real-time RT-PCR. Data obtained from matched cross-over samples ( $n = 7$  individuals) are plotted as a ratio of the gene of interest/GAPDH in arbitrary units as means  $\pm$  SEM. Significance was tested by non-parametric ANOVA (Friedman) with a Dunn's *post-test*. \* $P < 0.05$ .

often below, the detection limit of the PCR assay and no significant effects were noted (data not shown). CXCL8 mRNA expression was readily detectable and this appeared to be elevated in allergen-challenged placebo samples and decreased in the budesonide-treated group (Supporting Information Figure S1B). However, these effects did not reach significance, and no firm conclusions can be drawn. By contrast, whereas CCL5 expression was unaltered between the saline and the allergen-challenged placebo groups, budesonide treatment significantly reduced CCL5 expression when compared to allergen-challenged placebo (Supporting Information Figure S1B). Taken with the previously reported clinical effects (protection from allergen-induced reductions in provocative concentration causing a 20% fall in FEV<sub>1</sub> and the reduction in intra-epithelial eosinophil numbers) (Kelly *et al.*, 2010), these data confirm the effectiveness of the ICS treatment.

### Corticosteroids induce GILZ expression in human airway epithelial and smooth muscle cells

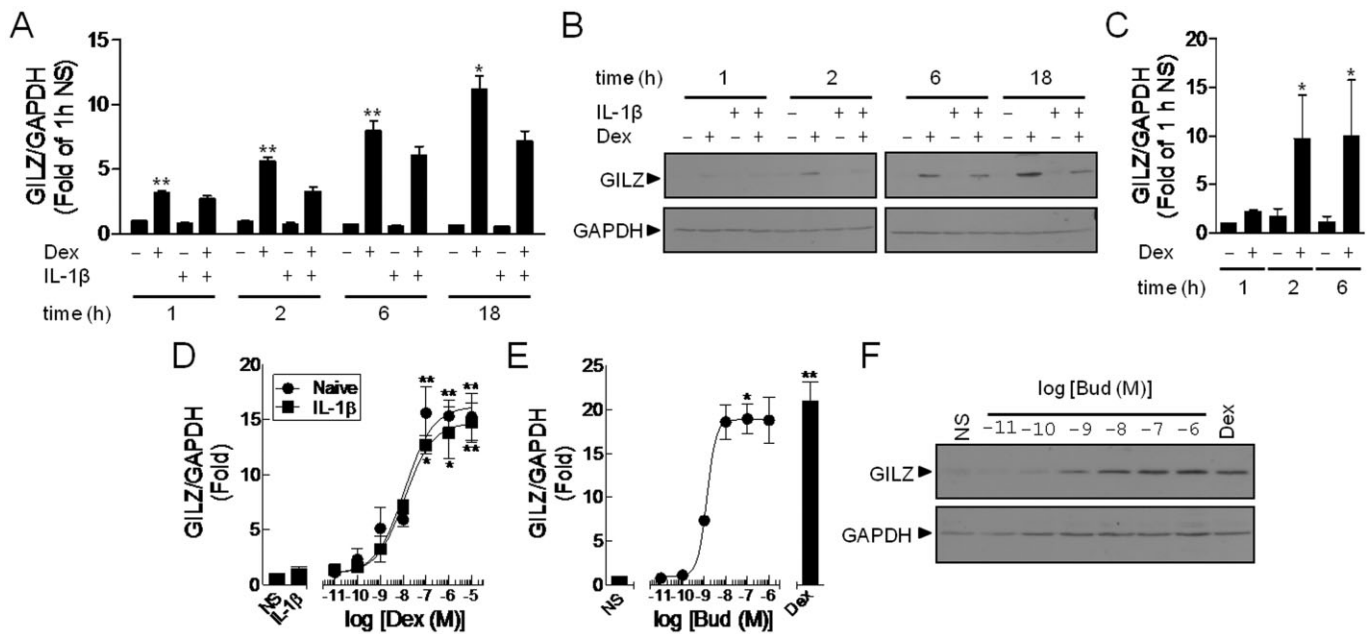
To test the credibility of the above result, the ability of glucocorticoids to induce GILZ expression was examined in epi-

thelial and smooth muscle cells, as these were previously shown to be present in the biopsy samples (Kelly *et al.*, 2010). Initially, A549 pulmonary epithelial cells were treated with maximally effective concentrations of dexamethasone and IL-1 $\beta$  (as a generic pro-inflammatory stimulus that would be produced following activation of allergen-stimulated antigen-presenting cells). GILZ mRNA was rapidly, within 1 h, up-regulated by dexamethasone, and this effect increased over 18 h (Figure 2A). Likewise, expression of GILZ protein was modestly, but significantly, increased 2 h post-dexamethasone treatment (Figure 2B and Supporting Information Figure S2A). GILZ levels then increased with robust, and significant, expression detected at 6 and 18 h. In each case, IL-1 $\beta$  had no effect on basal GILZ mRNA or protein expression but showed a trend towards reducing the increased GILZ mRNA and protein expression induced by dexamethasone. While this repressive effect of IL-1 $\beta$  failed to reach significance at any time tested, the presence of IL-1 $\beta$  did, however, prevent the induction, by dexamethasone, of GILZ mRNA and protein from reaching significance at all time points (Figure 2A & B, Supporting Information Figure S2A). Primary HBE cells were also treated with dexamethasone, and GILZ mRNA expression was examined (Figure 2C). GILZ mRNA was significantly up-regulated by dexamethasone at 2 and 6 h, and this confirms the utility of the A549 cell model. To examine the sensitivity of GILZ expression to dexamethasone, A549 cells were treated with various concentrations of dexamethasone and GILZ mRNA expression evaluated (Figure 2D). GILZ mRNA was found to be concentration-dependently increased by dexamethasone ( $EC_{50} = 1.2 \times 10^{-8}$  M). This was essentially unaffected by the presence of IL-1 $\beta$ . In the presence of increasing budesonide concentrations, GILZ mRNA expression was increased with an  $EC_{50}$  of  $1.3 \times 10^{-9}$  M (Figure 2E). Qualitatively similar data were obtained by Western blotting, and densitometric analysis produced an  $EC_{50}$  of  $3.8 \times 10^{-9}$  M (Figure 2F & Supporting Information Figure S2B). Thus, GILZ expression is readily detected at low nM concentrations of budesonide.

### Corticosteroids induce GILZ expression in primary human ASM cells

Following treatment of primary human ASM cells with dexamethasone, GILZ mRNA was significantly increased at all times tested relative to untreated control samples (Figure 3A). In similar experiments, budesonide significantly increased GILZ protein expression at 6 and 18 h (Figure 3B & Supporting Information Figure S3A). This effect was not apparent at 1 h, and at 2 h, GILZ protein expression was variable. To examine the sensitivity of this GILZ induction, ASM cells were treated with various concentrations of budesonide. Cells were harvested after 6 h, and GILZ expression was examined by real-time RT-PCR and Western blotting. GILZ mRNA was significantly increased by budesonide in a concentration-dependent manner ( $EC_{50}$  of  $2.9 \times 10^{-10}$  M) (Figure 3C). A similar result was evident by Western blotting, and densitometric analysis of data from 10 individuals revealed an  $EC_{50}$  of  $2.1 \times 10^{-10}$  M (Figure 3D & Supporting Information Figure S3B).





**Figure 2**

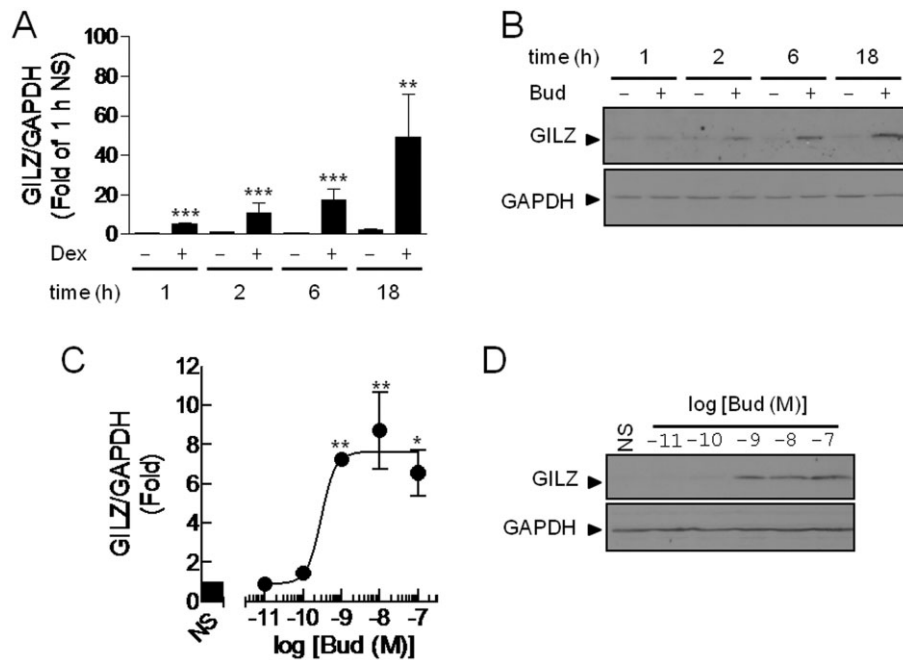
GILZ expression is induced by corticosteroids in A549 pulmonary cells and primary human bronchial epithelial cells. (A) A549 cells were treated with combinations of IL-1 $\beta$  (1 ng·mL<sup>-1</sup>) and dexamethasone (1  $\mu$ M) (Dex). Cells were harvested at the times indicated for real-time RT-PCR analysis of GILZ and GAPDH mRNA. Data ( $n = 7$ ), as a ratio of GILZ/GAPDH, are expressed as fold relative to non-stimulated (NS) at 1 h and are plotted as means  $\pm$  SEM. Significance, relative to NS and between groups, was tested at each time by paired non-parametric ANOVA (Friedman) with a Dunn's post-test. (B) Cells treated as in (A) were harvested for total protein and Western blotting was performed for GILZ and GAPDH. Images are representative of seven such blots (See Supporting Information Figure S2A for densitometric analysis). (C) Primary human bronchial epithelial cells (HBE) cells were treated with dexamethasone (1  $\mu$ M) (Dex) for the indicated times. RNA was extracted for real-time RT-PCR analysis of GILZ and GAPDH. Data ( $n = 4$ ) are plotted as in (A). Significance, relative to NS at each time, was tested by non-parametric  $t$ -test (Mann-Whitney  $U$ ). (D) A549 cells were treated with various concentrations of dexamethasone in the absence and presence of IL-1 $\beta$  (1 ng·mL<sup>-1</sup>). After 6 h, RNA was extracted for real-time RT-PCR analysis of GILZ and GAPDH. Data ( $n = 6$ ), as a ratio of GILZ/GAPDH, are expressed as fold relative to NS and are plotted as means  $\pm$  SEM. EC<sub>50</sub> values of  $1.2 \times 10^{-8}$  M were obtained in both cases. (E) A549 cells were treated with various concentrations of budesonide or dexamethasone (1  $\mu$ M) (Dex) and cells were harvested after 6 h. RNA was extracted for analysis of GILZ and GAPDH mRNA expression using real-time RT-PCR. Data ( $n = 4$ ), as a ratio of GILZ/GAPDH, are expressed as fold relative to NS and are plotted as means  $\pm$  SEM. In (D) and (E), significance was tested relative to the no corticosteroid control by paired non-parametric ANOVA (Friedman) with a Dunn's post-test. (F) Cells treated as in (E), were subjected to Western blot analysis of GILZ and GAPDH. Blots shown are representative of seven such experiments (See Supporting Information Figure S2B for densitometric analysis). Significance is indicated where: \* $P < 0.05$ , \*\* $P < 0.01$ .

### Immunolocalization of GILZ expression in the airways of asthmatic subjects

Prior to immunolocalization analysis for GILZ in the biopsy samples, it was essential to validate the specificity of the GILZ antibody. Given the above *in vitro* data, A549 cells were treated with dexamethasone for 6 h in the presence of either a control siRNA or GILZ-targeting siRNA to selectively knock-down GILZ expression. Western blot analysis revealed robust dexamethasone-induced GILZ protein expression (Supporting Information Figure S4). This was unaffected by control siRNA but was virtually absent in the presence of GILZ-targeting siRNA. Similarly, immunohistochemistry revealed that horseradish peroxidase (HRP) activity (brown) was either not or only barely detected in untreated samples, whereas following dexamethasone treatment, a robust brown colour reaction was observed within the cytoplasm (Supporting Information Figure S4B & C). This response was unaltered by the control siRNA, but was abolished in the presence of the GILZ targeting siRNA. Likewise, A549 cells that had been transfected with a control plasmid revealed little or no GILZ

expression by Western blotting or immunohistochemistry (Supporting Information Figure S5). Following transfection with GILZ-expressing plasmid, GILZ expression was strongly detected by Western blotting, and 10–15% of the cells revealed a strong positive HRP colour reaction (Supporting Information Figure S5). These data unequivocally validate the specificity of the GILZ antibody.

In the biopsy samples, immunolocalization showed GILZ protein to be expressed in the airway epithelium, smooth muscle and scattered inflammatory cells in the airway wall (Figure 4A–C). This staining was diffuse within the cytoplasm of the epithelium. In placebo-treated post-allergen challenge samples, much of the epithelium was only modestly stained (Figure 4A). This compares with the budesonide-treated group, which, post-allergen, showed a markedly elevated expression of GILZ in the epithelium (Figure 4B). Using a blinded semi-quantitative scoring system, GILZ immunoreactivity was shown to be significantly enhanced in the epithelium of budesonide-treated biopsies relative to placebo controls (Figure 4D). Thus, inhaled budesonide increased epi-



**Figure 3**

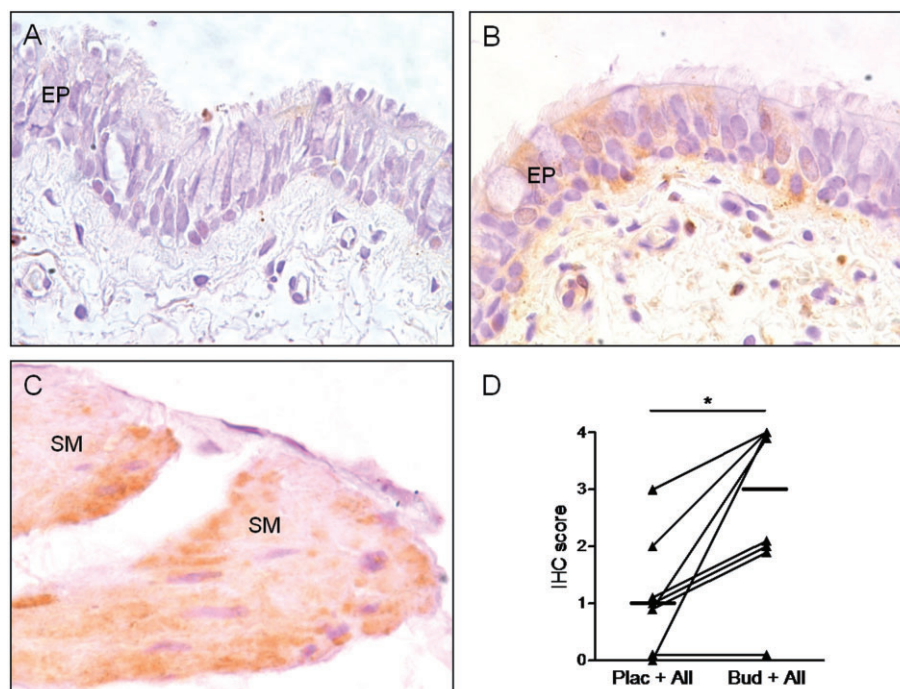
GILZ expression is induced by corticosteroid in primary human airway smooth muscle (ASM) cells. (A) ASM cells were treated with dexamethasone (1  $\mu$ M) (Dex). Cells were harvested at the times indicated for real-time RT-PCR analysis of GILZ and GAPDH. Data ( $n = 6-7$ ), as a ratio of GILZ/GAPDH, are expressed as fold of non-stimulated (NS) cells at 1 h and are plotted as means  $\pm$  SEM. Significance, relative to untreated at each time point, was performed by non-parametric *t*-test (Mann-Whitney *U*). (B) ASM cells were treated with budesonide (0.1  $\mu$ M) (Bud) and harvested at the times indicated for Western blot analysis of GILZ and GAPDH. Blots shown are representative of six such experiments (See Supporting Information Figure S3A for densitometric analysis). (C) ASM cells were either NS or treated with various concentrations of budesonide. Cells were harvested after 6 h for real-time RT-PCR analysis of GILZ and GAPDH. Data ( $n = 4$ ), as a ratio of GILZ/GAPDH, are expressed as fold relative to NS cells and are plotted as means  $\pm$  SEM. Significance was tested by paired non-parametric ANOVA (Friedman's) with a Dunn's post-test. (D) ASM cells from experiments as in (C), were harvested for Western blot analysis of GILZ and GAPDH. Images are representative of 10 such blots (See Supporting Information Figure S3B for densitometric analysis). Significance is indicated where: \* $P < 0.05$ , \*\* $P < 0.01$  or \*\*\* $P < 0.001$ .

thelial GILZ expression from that observed in the placebo group. GILZ staining in the ASM was patchy and variable with some muscle being faintly stained, while other areas were strongly stained (Figure 4C). This being the case, it was not possible to unambiguously state that GILZ staining in the ASM was changed with any of the interventions.

## Discussion

ICS represent a highly effective and widely prescribed therapy for the treatment of asthma (Barnes, 2006). However, while there is considerable literature detailing the ability of corticosteroids to act on GR and directly transrepress key inflammatory transcription factors (such as NF- $\kappa$ B and AP-1), this mechanism does not readily explain the ability of transcriptional, or translational, inhibitors to prevent the corticosteroid-dependent repression of inflammatory gene expression. Instead, such data suggest that corticosteroids induce the expression of anti-inflammatory genes to elicit anti-inflammatory effects (Stellato, 2004; Clark, 2007; Newton and Holden, 2007). Support for this contention has been incrementally accumulating and we now know that corticosteroids induce the expression of hundreds of genes

(Newton and Holden, 2007). While the biological functions of most of these genes remain to be unequivocally determined, there are notable examples that amply illustrate the anti-inflammatory potential of such a mechanism. Thus, corticosteroids rapidly and profoundly induce the expression of MKP-1 in a wide variety of tissues and cell types, and by switching off MAPK pathways, which are critical to both inflammation and the expression of many inflammatory genes, MKP-1 can exert anti-inflammatory activity (Clark *et al.*, 2008). This point is confirmed by findings that animals (or cells) lacking MKP-1 reveal deficits in, although not a total lack of, the repressive effects of corticosteroids (Abraham *et al.*, 2006; Furst *et al.*, 2007; Maier *et al.*, 2007). Importantly, such gene deletion studies are only likely to result in partial phenotypes since the ability of corticosteroids to induce multiple anti-inflammatory genes indicates functional redundancy (Newton and Holden, 2007). For example, the inhibition of NF- $\kappa$ B-dependent transcription by corticosteroids can, independently, be attenuated by GILZ (Di Marco *et al.*, 2007; Eddleston *et al.*, 2007), inhibitor of  $\kappa$ B $\alpha$  (NFKBIA) (Scheinman *et al.*, 1995), and potentially MKP-1 (King *et al.*, 2009), such that the loss of any single gene has the potential to be masked by the continued expression of the others (Newton and Holden, 2007).



**Figure 4**

GILZ protein is expressed in the airways of asthmatic subjects. Biopsy samples were subjected to immunohistochemical detection of GILZ protein using a validated antibody (See Supporting Information Figures S3 and S4). Samples depicted are: (A) post-allergen challenge after treatment with placebo; (B) post-allergen challenge after treatment with budesonide; and (C) post-allergen challenge after treatment with budesonide. Magnification: (A) 400 $\times$  (B, C) 600 $\times$ . (D) Comparison of staining for GILZ protein in bronchial epithelium from eight subjects obtained, post-allergen challenge (All) after treatment with placebo (Plac) or budesonide (Bud). GILZ immunoreactivity staining was scored according to five categories where: 0 = no staining; 1, <25%; 2, 25–50%; 3, 50–75% and 4, >75% staining. Individual immunohistochemical (IHC) scores are plotted and medians are indicated as horizontal bars. Significance was tested by Wilcoxon signed rank test. \* $P < 0.05$ . Note: where symbols and lines would have overlapped, data have been adjusted solely to facilitate visualization. EP = epithelium, SM = smooth muscle.

However, despite the increasing volume of data supporting anti-inflammatory roles for corticosteroid-induced genes, there is currently no direct evidence that such genes can be induced in asthmatic patients taking ICS. Rather, analysis of corticosteroid-induced transcription shows that induction of transcription from simple glucocorticoid response element (GRE)-dependent reporters occurs at higher steroid concentrations than is observed for repression of inflammatory gene expression (Jonat *et al.*, 1990; Chivers *et al.*, 2006). Such findings were always perceived as a major barrier in respect of the anti-inflammatory effects of corticosteroids being attributed to gene induction. Despite this, it is now clear that the simple GRE, as found in many GRE-dependent reporters, as a model for gene induction by corticosteroids probably only applies to a minority of the genes that are induced by corticosteroid, and that more complex interactions at composite GRE sites may govern gene induction in the majority of cases (So *et al.*, 2008). Thus, the induction of MKP-1 transcription by corticosteroids has been shown to occur via non-classical GRE sites, and this observation provides an explanation for the fact that MKP-1 is actually induced over a similar range of corticosteroid concentrations to that observed for the repression of inflammatory genes (King *et al.*, 2009; Tchen *et al.*, 2010). Likewise, both the GILZ and FKBP51 genes are associated with a number of GR-binding regions that were identi-

fied by chromatin immunoprecipitation (Wang *et al.*, 2004; So *et al.*, 2008). In each case, these sites reveal numerous mismatches with the classical GRE consensus (So *et al.*, 2008), yet in the case of GILZ, for which there appear to be at least three functional GRE sites (Wang *et al.*, 2004), there is previously documented nanomolar sensitivity to dexamethasone (Chen *et al.*, 2006). Consequently, such corticosteroid-induced gene products do offer a plausible explanation for at least some of the anti-inflammatory actions of corticosteroids. However a lack of hard evidence to support this contention *in vivo* prevents progress in this advancing field. Therefore, we have taken advantage of biopsy samples obtained from asthmatics taking inhaled budesonide in an attempt to shed light on this key question.

To examine whether ICS induce gene expression in asthmatic patients, we analysed bronchial biopsy samples from a clinical study in which mild asthmatics were treated with inhaled budesonide (800  $\mu$ g daily) or placebo for 11 days (Kelly *et al.*, 2010). As previously reported, participants were subjected to a mild allergen challenge, the dose of which was two doubling doses below that predicted to achieve a 20% fall in FEV<sub>1</sub> (Kelly *et al.*, 2010). The mild nature of this challenge, as well as the mild asthma phenotype of the participants, provides an explanation for the, previously reported, lack of inflammatory cell influx to the lung (Kelly *et al.*, 2010). This

finding is also reflected in the lack of significant induction of inflammatory cytokines/chemokines, such as IL-1 $\beta$ , CXCL8 and CCL5, observed in the current study. Notwithstanding these data, Kelly *et al.* (2010) showed budesonide to exert clear therapeutic benefits and we now show a repressive effect on CCL5 gene expression. Therefore, in the present study, we used real-time RT-PCR to test for corticosteroid-induced gene mRNA expression of GILZ, MKP-1 and FKBP51 in the biopsy samples that had been taken after 10 days of inhaled budesonide. While the effect of the inhaled allergen challenge did not significantly affect basal expression of any gene, GILZ and FKBP51 were significantly up-regulated in the budesonide-treated samples. Since the biopsy samples used for gene expression analysis were obtained post-allergen challenge, there is a definite possibility that the presence of budesonide may have sensitized or enhanced the response to the allergen. However, both GILZ and FKBP51 are both firmly established as corticosteroid-inducible genes (Wang *et al.*, 2004; Chivers *et al.*, 2006; Kaur *et al.*, 2008b). Furthermore, since inflammatory stimuli such as IL-1 $\beta$  or TNF- $\alpha$  do not, on their own, induce GILZ expression or enhance corticosteroid-induced GILZ expression (but rather tend to reduce GILZ expression) (this study and Rider *et al.*, 2011), these data provide strong support for the contention that ICS induce expression of these genes. Despite a similar upward trend being described in respect of MKP-1, the lack of a significant enhancement of MKP-1 expression in the budesonide samples may be a reflection of the fact that MKP-1 is very rapidly and acutely up-regulated by corticosteroids and therefore may play a more significant role in the initial effects of corticosteroid treatment. Alternatively, MKP-1 is itself strongly up-regulated by inflammatory stimuli, and this could, due to the allergen challenge or the asthmatic phenotype, lead to confounding effects that mask the induction by corticosteroids. While MKP-1 is clearly up-regulated in cells from the lung, for example, alveolar macrophage (Bhavsar *et al.*, 2008), further investigation is required to assess the ability of ICS to induce MKP-1 *in vivo*. In this context, it is possible that the presence of lung inflammation could reduce glucocorticoid responses, as GRE-dependent transcription is reduced by inflammatory mediators in both epithelial and ASM cells (Tliba *et al.*, 2008; Rider *et al.*, 2011). Indeed, the current *in vitro* data presented support this possibility, as the ability of corticosteroid to induce GILZ expression was less robust (not significant) in IL-1 $\beta$ -treated cells. Equally, it is possible that other aspects of the asthma phenotype may lead to reduced corticosteroid responses as alveolar macrophages taken from severe asthmatics showed a reduced ability, compared with less severe disease, to induce MKP-1 in response to dexamethasone (Bhavsar *et al.*, 2008).

While the functional properties of GILZ, for example, in reducing NF- $\kappa$ B- and AP-1-dependent transcription (Mittelstadt and Ashwell, 2001; Eddleston *et al.*, 2007), or IL-8 expression (Eddleston *et al.*, 2007), are consistent with anti-inflammatory efficacy (Ayroldi and Riccardi, 2009), FKBP51 function is less well defined and may involve negative regulation of GR activity (Davies *et al.*, 2002; Stechschulte and Sanchez, 2011). Thus, it is possible that the induction of FKBP51 by ICS may represent a therapeutically unhelpful effect that will tend to reduce corticosteroid responsiveness. In the current study, we have consequently examined GILZ

expression *in vitro* to further investigate potentially anti-inflammatory responses to corticosteroids. GILZ expression was significantly induced by corticosteroids in pulmonary A549 and primary HBE cells, and this is consistent with previous findings (Chivers *et al.*, 2006; Eddleston *et al.*, 2007; Kaur *et al.*, 2008a). Importantly, low, nanomolar, concentrations of the corticosteroid were able to induce GILZ expression. Thus, in A549 cells, dexamethasone or budesonide increased the expression of GILZ with EC<sub>50</sub> values of  $1.2 \times 10^{-8}$  and  $1.3 \times 10^{-9}$  M, respectively. In ASM cells, GILZ expression was even more sensitive to budesonide with EC<sub>50</sub> values of  $2.9 \times 10^{-10}$  and  $2.1 \times 10^{-10}$  M being obtained for the induction of GILZ mRNA and protein, respectively. Reasons for the differential sensitivity in the induction of GILZ expression between A549 and ASM cells are not currently clear. However, such a result could be due to differential levels of GR expression or factors (e.g. transcriptional co-activators or other transcription factors) that enable GR to couple to the relevant downstream responses (in this case, GILZ transcription) (Newton *et al.*, 2010b). Such effects may be therapeutically significant as not all cells will respond to ICS in an equivalent manner.

As ICS concentrations of ~1–100 nM are readily achievable in the lung (Miller-Larsson and Selroos, 2002; Todorova *et al.*, 2006; van den Brink *et al.*, 2008), these data provide convincing support for the observed induction of GILZ in the biopsy samples. Thus, following inhaled doses of 1 or 1.6 mg budesonide, concentrations of up to 61.7 and 8.9 nmol·kg<sup>-1</sup> were variously described in the lung (van den Bosch *et al.*, 1993; van den Brink *et al.*, 2008). Similarly, plasma concentrations of budesonide, following a 1 mg inhalation using a Turbuhaler, were found to rapidly peak at 1–10 nM and then decline in the following hours (Thorsson *et al.*, 1994; van den Brink *et al.*, 2008). Given a relatively constant level of lung deposition ( $38 \pm 9\%$ ) achieved with the Turbuhaler over a range of inhaler doses (Borgstrom *et al.*, 2005), one might expect the current protocol to have achieved a twice-daily delivery of ~150  $\mu$ g budesonide (i.e. 0.35  $\mu$ mol). Simplistically, if distributed in a lung tissue volume of ~2 L, it is possible that budesonide concentrations in the order of 100 nM could be reached. However, non-uniform distribution, solubility, export in the blood and/or mucus, chemical modification and protein binding are factors that can act to reduce effective drug concentrations. Finally, ICS dosing occurs against a background of endogenous cortisol that will be present in these individuals due to normal diurnal variation and any stresses that they encounter. Thus, basal activation of GR *in vivo* will be greater than *in vitro*, and this will tend to reduce the overall fold change observed *in vivo*.

In terms of the cells targeted by ICS, it is possible that the presence of corticosteroid could, over the time frame of the study, lead to significant alterations to the cell populations that make up the biopsy, and this could potentially modify downstream outputs. However, immunohistochemical analysis of biopsies from the budesonide-treated group revealed GILZ protein expression in the airway epithelial cells and in the ASM. This is consistent with the above *in vitro* findings. In addition, we observed the occasional inflammatory cell showing a positive reaction to GILZ, and this is supported by previous findings showing that GILZ is expressed in inflammatory cells (Mittelstadt and Ashwell, 2001). Overall, the



budesonide-treated, allergen-challenged, group showed a similar pattern of GILZ expression (i.e. epithelial and ASM cells) as the saline challenge controls (data not shown). However, relative to the placebo-treated, post-allergen challenge group, the presence of budesonide significantly enhanced staining for GILZ in the epithelium (Figure 4D). While it is possible that the allergen challenge may have led to a reduction in epithelial GILZ (see earlier discussion), it is clear that inhaled budesonide increased GILZ expression in the epithelium. This is consistent with the mRNA expression data from the biopsies. However, immunohistochemistry is primarily a qualitative technique and is not well suited to assessing small changes in expression. Thus, a similar effect could not be demonstrated in the ASM, in part due to the variable intensity of staining within the groups.

In summary, these studies provide convincing evidence for the concept that corticosteroid-inducible genes, such as GILZ, are induced in the airways following ICS treatment. This demonstration means that the functional contribution of corticosteroid-induced genes to the anti-inflammatory response needs to be taken into account when considering the pharmacological properties of ICS. Confirmation that novel corticosteroids, or other GR-activating ligands, induce anti-inflammatory genes, such as GILZ or MKP-1, may provide a useful component to screening strategies for improved anti-inflammatory action by GR (Newton *et al.*, 2010b). Equally, not all the effects of corticosteroids are desirable when used as an anti-inflammatory therapy (Schacke *et al.*, 2002). This point is exemplified by the induction of FKBP51, which may act to dampen, or repress, the response to corticosteroids (Stechschulte and Sanchez, 2011). Consequently, our data should encourage the analysis of corticosteroid-induced genes as a key step towards further understanding and then optimizing or enhancing GR action (Newton *et al.*, 2010b). Such advances will probably have broad clinical implications for the way we treat asthma and other inflammatory lung diseases.

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## Conflicts of interest

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** mRNA expression of MKP-1, CXCL8 and CCL5 in bronchial biopsies from mild atopic asthmatics taking inhaled budesonide. Mild asthmatics were entered into a cross-over study that has been described previously (Kelly *et al.*, 2010). Patients were initially subjected to a 3 week washout period prior to an inhaled challenge with diluent alone (saline). Following this, patients were subjected to a further 3 week washout period prior to treatment with placebo (Plac) or drugs for 11 consecutive days with a cross-over design. Budesonide (Bud) was inhaled using a Pulmicort Turbuhaler (budesonide, 200 µg) with 2 inhalations taken twice daily and placebo was administered in a similar manner. On day 9, patients were subjected to allergen inhalation (All). Bronchoscopy was performed on day 10 (24 h after the allergen challenge). Biopsies were analysed for mRNA expression of GAPDH, MKP-1, CXCL8 and CCL5 using real-time RT-PCR. Data were obtained from matched samples from 9 individuals and are plotted as a ratio of the gene of interest/GAPDH in arbitrary units as means  $\pm$  SE. Significance between Plac + All and Bud + All was tested by Mann–Whitney test.  $*P < 0.05$ .

**Figure S2** GILZ protein expression is induced by corticosteroids in A549 pulmonary cells. (A) As described in Figure 2B of the main manuscript, A549 cells were treated with combinations of IL-1 $\beta$  (1 ng/mL) and dexamethasone (1 µM) (Dex) or were not treated. Cells were harvested at the times indicated for total protein and subsequent Western blot analysis of GILZ and GAPDH. Following densitometric analysis, data ( $n = 7$ ), as a ratio of GILZ/GAPDH, were expressed as fold over untreated at  $t = 1$  and are plotted as mean  $\pm$  S.E. Significance relative to untreated was tested at each time point by non-parametric ANOVA (Friedman's) with a Dunn's post-test. (B) As

described in Figure 2F of the main manuscript, A549 cells were either non-stimulated (NS) or treated with various concentrations of budesonide (as indicated) or dexamethasone (1 µM) for 6 h prior to harvesting for total protein. Western blotting was performed for GILZ and GAPDH. Following densitometric analysis, data ( $n = 7$ ), as a ratio of GILZ/GAPDH, were expressed as fold over NS and are plotted as mean  $\pm$  S.E. An EC<sub>50</sub> of  $3.8 \times 10^{-9}$  M was obtained for the induction of GILZ protein by budesonide. Significance relative to NS was tested by non-parametric ANOVA (Friedman's) with a Dunn's post-test. Significance is indicated where:  $*P < 0.05$ ,  $**P < 0.01$  or  $***P < 0.001$ .

**Figure S3** GILZ expression is induced by budesonide in primary human airway smooth muscle (ASM) cells. (A) As described in Figure 3B of the main manuscript, primary human ASM cells were treated with budesonide (0.1 µM) and harvested at the times indicated for Western blot analysis of GILZ and GAPDH. Following densitometric analysis, data ( $n = 6$ ), as a ratio of GILZ/GAPDH, were expressed as fold over untreated at 1 h and are plotted as mean  $\pm$  SE. Significance relative to untreated was tested at each time point by non-parametric paired *t*-test. (B) ASM cells were treated with various concentrations of budesonide as indicated for 6 h prior to harvesting for total protein. Western blotting was performed for GILZ and GAPDH. Following densitometric analysis, data ( $n = 10$ ), as a ratio of GILZ/GAPDH are expressed as fold over NS and are plotted as mean  $\pm$  SE. An EC<sub>50</sub> of  $2.1 \times 10^{-10}$  M was obtained for the induction of GILZ protein by budesonide. Significance relative to NS was tested by non-parametric ANOVA (Friedman's) with a Dunn's post-test. Significance is indicated where:  $*P < 0.05$  or  $**P < 0.01$ .

**Figure S4** siRNA-mediated knockdown of dexamethasone-induced GILZ. (A) A549 cells were incubated with control or GILZ-specific siRNA for 24 h prior to stimulation with dexamethasone (0.1 µM) for 6 h. Cell lysates were then subjected to Western blot analysis for GILZ and GAPDH. (B,C) In parallel experiments, performed at the same time as those in (A), A549 cells plated into 4-chamber slides were incubated in the presence of either control siRNA or GILZ-targeting siRNA for 24 h, prior to treatment with dexamethasone (0.1 µM). Cells were subjected to immunohistochemical analysis for GILZ. (B) Depicts representative images of untreated and dexamethasone-treated cells. (C) Representative images of dexamethasone-treated cells in the presence of either control siRNA or GILZ-targeting siRNA are shown. Magnification was either 20 $\times$  or 60 $\times$  10 as indicated.

**Figure S5** (A) A549 cells were either not transfected or transfected with control plasmid (pGL3) or GILZ expressing plasmid for 24 h. Untransfected cells were also treated, or not, for 6 h with dexamethasone (0.1 µM). Cell lysates were then subject to Western blot analysis for GILZ and GAPDH. In each case, blots representative of two such experiments are shown. (B) Cells treated as in (A) were analysed in parallel for GILZ expression by immunohistochemistry. Representative images showing GILZ expression in the presence of (i) control plasmid or (ii) GILZ expressing plasmid are shown.

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