

Assessment of signal transducer and activator of transcription 6 as a target of glucocorticoid action in human airway epithelial cells

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Summary

Background Activation of signal transducer and activator of transcription (STAT)6 by IL-4 and IL-13 is essential in many key epithelial responses in the asthmatic airway including expression of numerous chemokines, goblet cell differentiation and mucus production and expression of other allergic inflammatory genes. While these responses are all inhibited by glucocorticoids (GC) administered systemically or by inhalation, the inhibitory mechanisms are unknown.

Objective To test the hypothesis that GC suppress allergic responses by blocking IL-4-induced STAT6 signalling in airway epithelial cells.

Methods Western blotting and reporter gene assays were used to determine whether GC could inhibit STAT6 production, phosphorylation or nuclear translocation, or whether GC could affect STAT6 transcriptional activity in the BEAS-2B airway epithelial cell line.

Results Our results showed that GC had no inhibitory effect on the total cellular or nuclear levels of STAT6 or phospho-STAT6. GC did not inhibit transcription from three different STAT6-driven reporter constructs, indicating that GC also did not inhibit STAT6 function.

Conclusion We conclude that airway epithelial STAT6 is not the central target of GC in allergic inflammation and that the inhibitory effect of GC on STAT6-mediated IL-4- and IL-13-induced responses is exerted by targeting pathways distinct from STAT6.

Keywords airway epithelium, allergy, asthma, chemokine, eotaxin, glucocorticoids, IL-4, IL-13, signal transduction, STAT6

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Introduction

Activation of the transcription factor signal transducer and activator of transcription (STAT)6 in airway epithelium is a critical step towards establishment of the chronic allergic inflammatory state as is seen in asthma. Knockout analysis and gene promoter studies indicate that STAT6 is a master regulator of multiple epithelial-expressed genes involved in the allergic inflammatory process, including many chemokines (eotaxin-1 (CCL11, [1]), eotaxin-2 (CCL24, [2]), eotaxin-3 (CCL26, [3]), monocyte chemoattractant protein-1 (MCP-1/CCL2, [4]), macrophage-derived chemokine (MDC/CCL22, [2]), thymus- and activation-regulated chemokine (TARC/CCL17, [2]), alternative macrophage activation-associated CC chemokine (AMAC1/CCL18, [5]), cell surface receptors (CD23 [6], major histocompatibility complex (MHC) class II, IL-4 receptor α chain (IL-4R α [7]), vascular cell adhesion molecule-1 (VCAM-1 [8]), IL-13R α 2 [9], β ₃ integrin [10], P-selectin [11]), various inflammatory molecules (IgE [12], IL-4 [13], *Muc5ac* [14], *Clca3/Gob5* [14], 12/15-

lipoxygenase [15], 15-lipoxygenase-1 [16], IL-4-induced secreted protein (FISP [17]), found in inflammatory zone 1 (FIZZ1/resistin-like α [14, 18]) and FIZZ2 [18], lymphotoxin- α [19], trefoil factor 2 [20]), as well as genes involved in goblet cell differentiation [21] and mucus production [14]. STAT6 activation occurs as a result of a signal transduction cascade (reviewed in [22]) that is initiated by the cytokines IL-4 and IL-13, secreted by the abundant CD4⁺, T-helper 2 (Th2) cells [23, 24] found in the allergic airway. These two cytokines bind their cell-surface receptors [25], which then activate members of the Janus kinase (JAK) family, eventually culminating in the phosphorylation and activation of STAT6 [26]. Homodimerization of two phosphorylated STAT6 monomers and translocation to the nucleus are necessary for the binding of this transcription factor to the STAT6 consensus sequence [27] and initiation of STAT6-dependent gene expression.

STAT6-dependent gene expression in airway epithelial cells plays a significant role in the development of allergic inflammation in the lung. Many *in vitro* studies support the notion that STAT6 signalling in airway epithelium leads to expression of a variety of different chemokines; these chemokines, such as the eotaxin family and others, are responsible for extensive recruitment of eosinophils and basophils to the lung [28, 29]. Degranulation and release of

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inflammatory mediators from these cells, including peptide leukotrienes [30], toxic eosinophil cationic [31] and major basic proteins [32], contribute to the bronchospasm and histopathology characteristic of asthma [33]. A recent analysis of chemokine gene expression in whole-lung extracts from STAT6 knockout mice revealed that expression of a host of chemokine genes including eotaxin-1, eotaxin-2, eotaxin-3, MDC, TARC, I-309, MCP-1, MCP-2, MCP-3 and MCP-4 was absent [2]. Although the specific cellular source of the chemokines was not established by this experiment, the authors speculated that the resident parenchymal cells, such as the airway epithelial cells and endothelial cells, would be the most likely candidates following activation by IL-4/IL-13 acting via Stat6. Consequently, STAT6 knockout mice did not exhibit experimental allergic inflammatory responses when sensitized and challenged by antigen inhalation. The promoters of these chemokine genes all contain STAT6 consensus sequence sites, suggesting that STAT6 may be centrally involved in the transcriptional regulation of chemokines necessary for recruitment and activation of inflammatory cells in allergic responses. However, it is important to point out that in one *in vivo* study, which directly addressed the contribution of STAT6 signalling in airway epithelial cells to the development of allergic inflammation, expression of STAT6 solely in airway epithelial cells was shown to be vital for development of both IL-13-induced airway hyper-responsiveness (AHR) and mucus production [14]. Interestingly, expression of transgenic STAT6 in epithelial cells alone did not restore some of the other features of IL-13-induced airway inflammation, such as inflammatory cell infiltration, fibrosis or production of the chemokine, MCP-1 [14]. Despite the results of this study, numerous *in vivo* studies have demonstrated that airway epithelium is a major source of chemokines involved in asthma pathogenesis. For example, airway sections from both mice and humans stained for STAT6-dependent chemokines, like eotaxin, by a variety of different methods clearly show that epithelial cells are the predominant cell type that express these molecules [34–36].

Besides expression of many chemokines, development of AHR and mucus production, STAT6 is also intimately involved in transcription of the IL-4R α chain [13], IgE [37] and the low-affinity IgE receptor, CD23 [38], as well as in the development and differentiation of Th2 cells [39]. The observation that STAT6 knockout mice show no AHR, lung eosinophilia or development of mucus cells upon allergen challenge suggests that STAT6 is important in tissue structural cells as well as in Th2 cells.

Asthma symptoms can be effectively managed by inhaled glucocorticoid (GC), which are the most important class of drugs presently used in asthma therapy to date (reviewed in [40]). In particular, GC treatment potently inhibits the production of eotaxin-1 and many other STAT6-dependent chemokines from human airway epithelium [41–44]. GC mediate their anti-inflammatory actions by binding to the glucocorticoid receptor (GR), a member of the nuclear receptor transcription factor family. GC-activated GR utilizes multiple mechanisms, both transcriptional and post-transcriptional, to regulate gene expression in either a negative or positive manner. One mechanism by which GR influences gene expression is by direct physical association with other transcription factors to affect their function, the most well-

studied being the nuclear factor κ B (NF- κ B) and AP-1 families [45]. GR can also interact with members of the STAT family in both a positive [46] and negative [47] way, and a physical interaction between STAT6 and the GR has been noted in primary human T cells [48]. Whether GR physically interacts with STAT6 in airway epithelial cells to regulate IL-4-induced gene expression is unknown.

Because STAT6 plays a central role in allergic inflammation and since allergic inflammatory responses such as leucocyte recruitment and goblet cell hyperplasia are well inhibited by GC, determination of the importance of STAT6 as a GC target is an important goal. In this study, we used western blotting techniques to investigate the phosphorylation and subcellular localization of STAT6 following GC treatment in human airway epithelial cells. Additionally, transient transfection studies were used to examine the influence of GC on the transcriptional function of STAT6. Here, we report that GC fail to suppress any marker of STAT6 activation or function examined. We conclude that the inhibitory effect of GC on expression of the eotaxin family and other STAT6-driven genes in airway epithelial cells is mediated by mechanisms other than inhibition of the STAT6 pathway.

Materials and methods

Cell culture

BEAS-2B, a human bronchial epithelial cell line transformed with a hybrid adenovirus 12-simian virus 40 (a kind gift from Dr Curtis Harris, NCI, Bethesda, MD, USA), was cultured in Ham's F-12/Dulbecco's-modified Eagle's medium (GIBCO BRL, Gaithersburg, MD, USA) with 5% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin and 0.2% Fungizone[®] (GIBCO BRL) at 37 °C in 5% CO₂ humidified air.

Glucocorticoid treatment and cytokine stimulation of cultured cells

Cells were used for experiments when they reached 70% confluency. Twenty-four hours prior to GC treatment, the medium was changed to FBS-free medium in all experiments to eradicate any effects of serum components, such as cortisol, on the GC experiments. Cells were then treated with the GC, budesonide (BUD; Sigma, St Louis, MO, USA; stored as a 0.1 M stock in dimethyl sulphoxide (DMSO) and diluted into cell culture medium) or fluticasone propionate (FP; GlaxoSmithKline, Oxbridge, UK, stored as a 0.1 M stock in DMSO and diluted into cell culture medium) at 10⁻⁷ M or DMSO diluent control overnight (18–24 h). For analysis of phospho-STAT6 protein levels, cells were stimulated with 0.2 ng/mL recombinant human IL-4 (R and D Systems, Minneapolis, MN, USA) for 30 min, then harvested by trypsinization, washed with Hanks' balanced salt solution and collected by centrifugation at 300 $\times$ g. For all the transfection studies, cells were stimulated for 6 h with the same concentration of IL-4 that was used for the phospho-STAT6 protein analysis (0.2 ng/mL), except where noted, and then harvested for measurement of luciferase activity. For analysis of eotaxin-1 mRNA and protein levels and eotaxin-3

RNA levels, cells were stimulated for 18 h with 50 ng/mL IL-4 and were then harvested as described for the phospho-STAT6 protein analysis.

Transfection with signal transducer and activator of transcription 6-driven luciferase reporter constructs and signal transducer and activator of transcription 6 expression vectors

Three STAT6-driven luciferase reporter constructs were used to transfect BEAS-2B cells transiently: the first, designated '----N4' (TPU475, from Tularik Inc, South San Francisco, CA, USA), was derived from a composite C/EBP-STAT6 site in the human germline IgE promoter [27] in which the C/EBP sites had been mutated. We therefore refer to this plasmid as mut-(C/EBP)-N₄-luc. A second reporter, pEotx.1363, was derived from the human eotaxin-1 promoter as described elsewhere [1]. A third reporter, pEotx.M2, is the same as pEotx.1363 but it has had the NF-κB site eliminated by mutation [1]. The following expression vectors were also used: wild-type STAT6 expression vector, referred to as WT-STAT6 (described in [27], from Tularik Inc.) and the empty expression vector pcDNA3TM (Invitrogen, Carlsbad, CA, USA).

Transient transfections were performed in triplicate wells for each treatment condition as previously described [1] with the following modifications: 0.95 µg luciferase reporter plasmid with or without 0.05 µg WT-STAT6 expression vector or empty pcDNA3TM vector was used for transfection. This 20:1 reporter to expression vector ratio was chosen as a result of titration experiments (data not shown) to determine the least possible amount of expression vector required to give a reasonable signal enhancement of the IL-4-induced luciferase activity. Luciferase Cell Lysis Buffer (150 µL, Pharmingen, San Diego, CA, USA) was used to solubilize the cells and the protein concentration of the lysates was determined as for the whole-cell extracts described. The luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA) and a luminometer (Analytic Luminescence Laboratories, Sparks, MD, USA). The luciferase activity was normalized to the protein concentration of the lysates; the protein concentration assay is described in the next subsection. The normalized luciferase activity was expressed as the fold induction of luciferase activity above the DMSO control condition, which was given a value of one.

Whole-cell, cytoplasmic and nuclear extraction

Whole-cell extracts from BEAS-2B cells were made as described [49] with the following modifications: protease and phosphatase inhibitors added to the boiling lysis buffer included complete Mini protease inhibitor cocktail tablets (Roche Diagnostics, Basel, Switzerland; as per manufacturer's instructions), 1 mM sodium orthovanadate, 50 mM sodium fluoride and 2 µg/mL Pepstatin A. The protein concentration of cell extracts was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). The sample was boiled and then stored in SDS-PAGE loading buffer (25 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate, 5% β-mercaptoethanol, 25% glycerol, 0.01%

bromophenol blue) at -70 °C prior to use in SDS-PAGE. Nuclear and cytoplasmic extracts were prepared using the NE-PER[®] kit (Pierce); complete Mini protease inhibitor cocktail tablets, 50 mM NaF, 1 mM Na₃VO₄ and 2 µg/mL Pepstatin A were added to the cytoplasmic extraction reagent 1 (CER1) and nuclear extraction reagent (NER) solutions of the NE-PER[®] kit. The protein concentration determination and storage of nuclear and cytoplasmic extracts were carried out in the same manner as described for the whole-cell extracts.

Western blot analysis

Whole-cell, cytoplasmic and nuclear extracts were subjected to 7.5% Tris-Glycine gel electrophoresis (PAGERTM Gold Precast gels, BioWhittaker Molecular Applications, Rockland, ME, USA) and then transferred to Sequi-Blot polyvinylidene difluoride membranes (BioRad, Hercules, CA, USA). Membranes were blocked overnight with 4% bovine serum albumin (Fisher Scientific, Fernwood, NJ, USA) in phosphate-buffered saline with 0.1% Tween-20 (PBST) before incubation with either mouse anti-human STAT6 (BD Pharmingen Transduction Labs, Franklin Lakes, NJ, USA) or mouse anti-human phospho-STAT6 (BD Pharmingen Transduction Labs) for 2 h at room temperature, followed by washing with PBST, and then incubation with anti-mouse Ig antibody conjugated to horseradish peroxidase (Amersham, Piscataway, NY, USA) for 1 h. Enhanced chemiluminescent substrate (ECL Western blot detection system; Amersham) was added after further washing with PBST and the membrane was exposed to film.

Northern blot analysis, enzyme-linked immunosorbent assay and real-time polymerase chain reaction

Northern blotting was performed on a 1% agarose/formaldehyde gel using 10 µg of total RNA (isolated using the RNeasy RNA isolation kit with DNase treatment (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions) essentially as previously described [50]. ELISA was performed as previously described [50].

For quantitative real-time PCR analysis of eotaxin-3 mRNA levels, 500 ng total RNA was reverse transcribed and 5 µL of a 1:10 dilution of the resulting cDNA was analysed by LUX primer quantitative real-time PCR for the presence of eotaxin-3 message using a pair of eotaxin-3-specific primers (a kind gift from Brian Lowe, Invitrogen) at 200 nM final concentration. The sequence of the FAM-labelled LUX forward primer was: 5'-CAC AGC CTC TCC TTG GCC TCT GCT G FAM G-3' and the sequence of the reverse primer was: 5'-GCT TGT GGC TGT ATT GGA AGC A-3' (synthesized by Invitrogen). The same cDNA samples were also analysed for the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to allow for normalization of the target eotaxin-3 sequence for the amount of input cDNA. The sequences of the specific GAPDH primers were as follows: for the forward primer, 5'-GGCATC CTG GGC TAC ACT GA-3' and the sequence of the JOE-labelled LUX reverse primer was: 5'-GAC CAC GGA AAT GAG CTT GAC AAA GTG G JOE C-3'; the master reaction mixture was made up using Platinum[®]

Quantitative PCR SuperMix-UDG according to the manufacturer's instructions (Invitrogen). These instructions were also followed to carry out the thermal cycling protocol for PCR tubes/plates and the analysis was performed using the ABI 7700 real-time PCR machine (Applied Biosystems, Foster City, CA, USA). The amount of eotaxin-3 mRNA in each sample was calculated according to the $2^{-\Delta\Delta C_t}$ method [51]. Briefly, the threshold value (or C_t) for the target sequence was normalized to the value obtained for the house-keeping gene and then that value was expressed as a relative change (compared with a control or unstimulated sample) in the level of target gene expression.

Software and statistical analysis

Bands detected by Western blotting on the autoradiographic film were captured digitally (Kodak 1D Image Analysis Software version 3.5) and band density was quantitated using Scion Image 1.62c. Statistical analysis was performed using paired *t*-tests with Statview version 5.0 and Kaleidagraph 3.5.2.

Results

Glucocorticoid effects on signal transducer and activator of transcription 6 protein

GC pre-treatment has been shown to inhibit the expression of many IL-4-induced genes and the activation of STAT6 is a critical step in the signal transduction pathway initiated by IL-4. Therefore, we determined whether GC pre-treatment of BEAS-2B cells decreased the levels of total cellular STAT6 protein. BEAS-2B cells were pre-treated with 10^{-7} M BUD for 18 h or DMSO diluent control and then stimulated with 0.2 ng/mL IL-4 for 30 min. This particular concentration of IL-4 was chosen as a result of IL-4 dose-response studies that measured the nuclear-localized phospho-STAT6 band density over a range of concentrations from zero to 100 ng/mL IL-4. The IL-4 concentration that resulted in 50% of the maximum phospho-STAT6 band density was 0.2 ng/mL IL-4 and at this concentration, any effect of the BUD pre-treatment on IL-4-induced phospho-STAT6 would be visible by changes in the phospho-STAT6 band density. The concentration of DMSO was equivalent in all treatment conditions (0.0001%) to eliminate any DMSO effects on signalling. Following IL-4 stimulation, whole-cell lysates were analysed by western blot with either anti-human STAT6 or anti-human phospho-STAT6 antibodies. The STAT6 blot (Fig. 1a, left) and the corresponding densitometric analysis (Fig. 1b) showed that there was no change in the overall level of cellular STAT6 in the cells under any of the treatment conditions. We performed a similar Western blot analysis to determine whether GC pre-treatment decreased the levels of total cellular phospho-STAT6. There was low phospho-STAT6 in the DMSO diluent control cell lysates, and a dense phospho-STAT6 band appeared using whole-cell lysates from IL-4-treated cells (Fig. 1a, right). In multiple experiments, BUD did not affect IL-4-induced STAT6 phosphorylation as determined by western blotting (Fig. 1a, right) and densitometry (Fig. 1c). These results suggest that GC do not

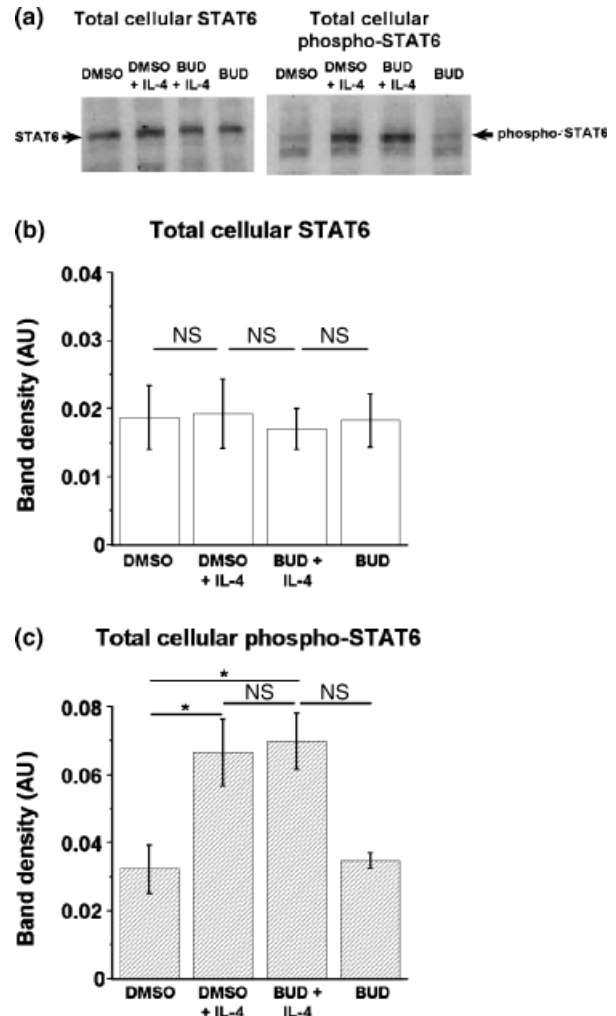


Fig. 1. Effect of budesonide (BUD) treatment on the levels of whole-cell signal transducer and activator of transcription (STAT)6 or whole-cell phospho-STAT6 in BEAS-2B cells. Cells were pre-treated for 18 h at 70% confluence with either dimethyl sulphoxide (DMSO) diluent control or 10^{-7} M BUD and then stimulated for 30 min with 0.2 ng/mL IL-4 and processed for western analysis as described in the Materials and methods. (a) Representative western blots showing total cellular STAT6 (left panel) and total cellular phospho-STAT6 (right panel) protein levels in BEAS-2B cells treated as indicated. (b) Densitometric analysis of STAT6 levels. Values are shown as means $\pm$ SEM of the densitometric analysis from six independent experiments. Arbitrary units (AU) of band density as measured by Scion Image 1.62c. (c) Densitometric analysis of phospho-STAT6. Data are shown as the mean $\pm$ SEM of the densitometric analysis from three independent experiments. AU of band density as measured by Scion Image 1.62c. * P < 0.05, NS (P > 0.05).

influence the total cellular levels of STAT6 nor do they inhibit the levels of phosphorylated STAT6 that result from the stimulation with IL-4.

To test the possibility that BUD might affect the STAT6 levels in the nuclear compartment, i.e. by effects on nuclear translocation, nuclear dephosphorylation, etc., we next analysed the STAT6 and phospho-STAT6 levels of nuclear extracts from treated cells by western blot. The blots and densitometric analysis (Fig. 2a, left and b) showed that there was detectable STAT6 in the nucleus of both DMSO diluent control and BUD-treated cells. Upon stimulation of the cells with 0.2 ng/mL IL-4, a significant increase in the level of STAT6 was detected in the nucleus as expected. This increase

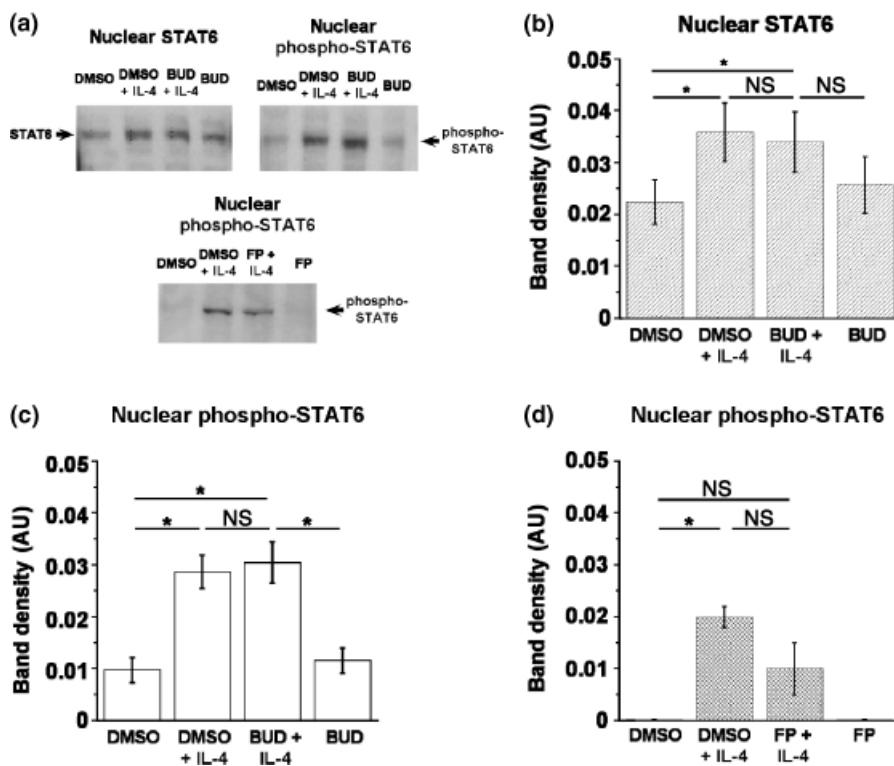


Fig. 2. Effect of budesonide (BUD) or fluticasone propionate (FP) treatment on the levels of nuclear-localized signal transducer and activator of transcription (STAT)6 and phospho-STAT6 in human airway epithelial cells. BEAS-2B cells were treated as indicated and then nuclear extracts were prepared and subjected to western analysis with anti-STAT6 or anti-phospho-STAT6 monoclonal antibodies. (a) Representative western blots showing total nuclear STAT6 (upper left panel) and nuclear phospho-STAT6 (upper right panel and lower panel) protein levels in BEAS-2B nuclear lysates treated as indicated. (b) Effect of BUD treatment on the levels of nuclear STAT6 in human airway epithelial cells. Data are shown as the mean $\pm$ SEM of the densitometric analysis from seven independent experiments. Arbitrary units (AU) of band density as measured by Scion Image 1.62c. * $P < 0.05$, ** $P < 0.01$, NS ($P > 0.05$). (c) BUD treatment had no effect on the levels of nuclear phospho-STAT6 in human airway epithelial cells. Data are shown as the mean $\pm$ SEM of the densitometric analysis from 12 independent experiments for BUD. AU of band density as measured by Scion Image 1.62c. * $P < 0.001$, NS ($P > 0.05$). (d) FP treatment had no effect on the levels of nuclear phospho-STAT6 in human airway epithelial cells. Data are shown as the mean $\pm$ SEM of the densitometric analysis from three independent experiments for FP. AU of band density as measured by Scion Image 1.62c. * $P < 0.01$, NS ($P > 0.05$).

in STAT6 protein in the nucleus was not prevented by 24 h pre-treatment with 10^{-7} M BUD prior to stimulation with IL-4.

We next determined whether the levels of phosphorylated STAT6 present in the nucleus were influenced by GC treatment. As expected, DMSO diluent control and BUD-treated cells had low levels of phospho-STAT6 protein present in the nucleus in the absence of IL-4 stimulation. Although stimulation with IL-4 significantly increased the levels of nuclear-localized phospho-STAT6 detected by Western blot (Fig. 2a, right), BUD pre-treatment did not prevent the increase of nuclear phospho-STAT6 induced by IL-4 (Fig. 2c). To be sure that the lack of inhibition of STAT6 activation was not peculiar to BUD, we tested the potent inhaled GC, FP, at a concentration of 10^{-7} M. This concentration was chosen because it has been shown to inhibit maximally epithelial expression of VCAM-1 induced by TNF- α in BEAS-2B cells [52]. Similar results were obtained when FP was used to treat the cells prior to IL-4 stimulation: FP pre-treatment did not prevent the increase of nuclear phospho-STAT6 induced by IL-4 (Fig. 2d).

Glucocorticoid effects on eotaxin-1 and eotaxin-3 protein and mRNA expression

Since there did not appear to be a detectable effect of BUD on IL-4-induced phospho-STAT6 levels in total cell lysates or in the nuclear fraction of treated cells, we sought to verify that the BUD and IL-4 stock solutions used for the aforementioned STAT6 protein analyses were functional and active. Stellato et al. [41] had shown previously that BUD pre-treatment of human airway epithelial cells had an inhibitory effect on IL-4-induced eotaxin-1 mRNA and protein levels. We used the same methods to examine eotaxin-1 mRNA and

secreted protein, as well as eotaxin-3 mRNA expression, to ensure that both the BUD and the IL-4 used in our studies were functional. As shown in Fig. 3a, BUD potentially inhibited IL-4-induced eotaxin-1 protein secretion and BUD also inhibited IL-4-induced expression of eotaxin-1 mRNA as shown by northern blot analysis and densitometric quantitation (Fig. 3b). These results repeated the findings of Stellato et al. and thereby demonstrate that the BUD and the IL-4 stock solutions used in our studies were active and functional. Furthermore, induction of another STAT6-dependent chemokine gene, eotaxin-3, by IL-4 was also significantly inhibited at the mRNA level by BUD pre-treatment as shown by quantitative real-time PCR analysis (Fig. 3c). These results confirm similar observations by Banwell et al. [53], which showed that IL-4- or IL-13-induced eotaxin-3 mRNA expression from the human lung epithelial cell line, NCI-H727, could be dramatically inhibited by 24 h pre-treatment with 1 μ M dexamethasone (DEX).

Glucocorticoid effects on signal transducer and activator of transcription 6 transcriptional function

Despite the lack of a decrease in the levels of nuclear-localized phospho-STAT6 in the BUD-treated/IL-4-stimulated cells, it was still possible that GC could be interfering with or inhibiting the transcriptional function of STAT6 in the nucleus. To test this possibility, three different STAT6-driven luciferase reporter gene constructs were transiently transfected into BEAS-2B cells and the cells were treated with BUD and/or IL-4. As we have previously reported, co-transfection with a wild-type STAT6 expression vector, WT-STAT6, was used to amplify the luciferase reporter gene activity in the BEAS-2B cells. Control experiments were carried out using the empty pcDNA3 expression vector in

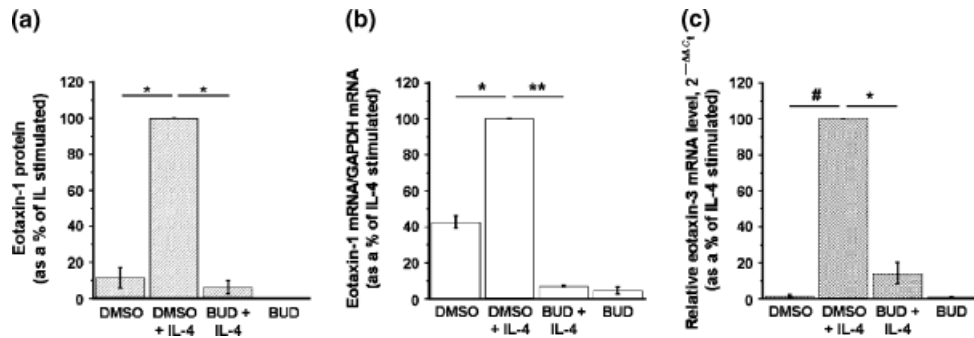


Fig. 3. Inhibition of the expression of eotaxin-1 protein and mRNA and eotaxin-3 mRNA by budesonide (BUD) treatment in BEAS-2B cells. Cells were treated at 70% confluence with 50 ng/mL IL-4 for 18 h, following 24 h pre-treatment with either 10^{-7} M BUD or dimethyl sulphoxide (DMSO) diluent control (DMSO); supernatants were harvested and analysed for eotaxin-1 by ELISA (a, $n = 3$) and RNA was harvested for northern blotting analysis (b, $n = 3$) and quantitative real-time PCR (c, $n = 3$). (a) ELISA data are expressed as the mean $\pm$ SEM eotaxin-1 protein as a percentage of the IL-4-stimulated value, which was 90 ± 50 pg/million cells. The mean $\pm$ SEM level of eotaxin-1 protein expressed by DMSO-treated BEAS-2B cells was 14 ± 8 pg/million cells. (b) Northern blot data are expressed as the densitometric analysis of eotaxin-1 mRNA expression normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. (c) Real-time PCR data are expressed as mean $\pm$ SEM eotaxin-3 mRNA levels, as a percentage of the IL-4-stimulated eotaxin-3 mRNA level. * $P < 0.05$, ** $P < 0.01$, # $P < 0.001$.

place of WT-STAT6 to ensure that the vector plasmid itself had no transactivating effects on the reporter plasmids.

One plasmid used, mut-(C/EBP)-N₄-luc, contains a tetramer of STAT6 sites derived from the I ϵ promoter and has been used extensively to monitor STAT6 transcriptional activity [27, 54–57]. Reporter activity was significantly induced (28-fold) with IL-4 treatment for 6 h in the presence of the WT-STAT6 expression vector (Fig. 4a, dark grey bars). BUD treatment did not decrease the luciferase reporter gene activity induced by IL-4. In fact, luciferase activity was slightly increased (1.6-fold above the IL-4-stimulated condition) in the presence of BUD and IL-4. DMSO diluent control and BUD alone did not induce luciferase activity from the reporter construct in the absence of IL-4. The positive control plasmid, pGL2-Control, induced the maximum luciferase activity (~ 98 -fold compared with mut-(C/EBP)-N₄-luc in DMSO-treated cells) and the negative control, pGL2-Basic, showed virtually no luciferase activity (Fig. 4, middle). As reported previously, omission of the co-transfected STAT6 resulted in modest activation of the reporter used [1]. However, the pattern of luciferase induction observed was identical to that shown in Fig. 4 (data not shown).

For most of the transfection experiments, BUD was added for 6 h after transfection to avoid any influence of GC on the transfection itself. In companion transfection experiments, transfected cells were subjected to a longer 18 h BUD pre-treatment, followed by IL-4 stimulation, to reproduce the pre-treatment conditions of the western blot experiments in Fig. 1. The luciferase data obtained from these 18 h BUD pre-treatment experiments had an identical pattern of induction of luciferase activity from the mut-(C/EBP)-N₄-luc plasmid (Fig. 4, right) to the pattern obtained with the 6 h BUD pre-treatment experiments (Fig. 4, left).

Two luciferase reporter plasmids derived from the eotaxin-1 promoter were also tested in this study in transiently transfected BEAS-2B cells as described. The first, pEotx.1363, is a 1363-bp fragment of the eotaxin-1 promoter (-1363 to -1), which has been shown to be activated by IL-4 treatment and contains a STAT6 site essential for transcription from the promoter, as shown by mutational studies and co-transfection with a dominant-negative STAT6 expression vector [1]. This 1363-bp fragment of the eotaxin-1 promoter also

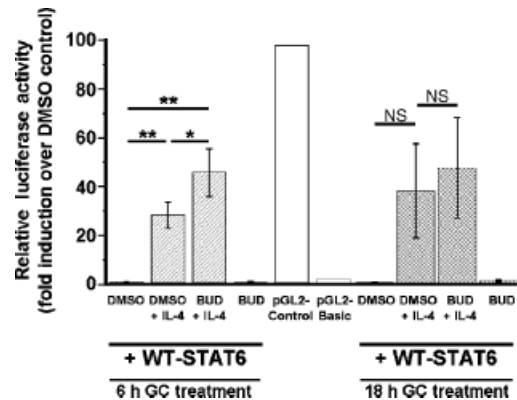


Fig. 4. Lack of inhibition of signal transducer and activator of transcription (STAT6)-mediated transcription from the STAT6-driven reporter plasmid, mut-(C/EBP)-N₄-luc by budesonide (BUD). BEAS-2B were co-transfected with mut-(C/EBP)-N₄-luc and a vector expressing wild-type STAT6 (WT-STAT6, as indicated by the horizontal bar) or they were transfected with control vectors (pGL2-Control and pGL2-Basic) overnight; then, triplicate samples were treated for 6 h (left, striped bars) in the presence of either dimethyl sulphoxide (DMSO) diluent control or 10^{-7} M BUD, with or without 0.2 ng/mL IL-4 stimulation. Transfected cells were also subjected to an 18 h pre-treatment protocol (right, hatched bars) with BUD or DMSO and then stimulated with or without 0.2 ng/mL IL-4 for 6 h. Luciferase activity was calculated as described in Materials and methods. Data are shown as mean induction of the luciferase activity above the DMSO control level $\pm$ SEM of five independent experiments. * $P < 0.05$, ** $P < 0.01$, NS ($P > 0.05$).

includes the glucocorticoid response element (GRE) described by the 1997 Garcia-Zepeda et al. study, which reports the cloning of the human eotaxin-1 gene. The second, pEotx.M2, is pEotx.1363 in which an NF- κ B site that overlaps the STAT6 site in the promoter has been mutated such that the promoter is rendered unresponsive to TNF- α . When pEotx.1363 was co-transfected with WT-STAT6, luciferase levels were induced to ~ 6.5 -fold above control with IL-4 stimulation (Fig. 5a, left). As was the case for the mut-(C/EBP)-N₄-luc reporter, BUD treatment did not prevent the IL-4-induced increase of luciferase activity in one experiment. The second eotaxin-1-derived luciferase reporter construct, pEotx.M2, showed a fivefold induction of luciferase activity over DMSO control with IL-4 stimulation (Fig. 5a, right), which was not significantly reduced in the BUD-treated cells. There was no significant induction of luciferase activity from

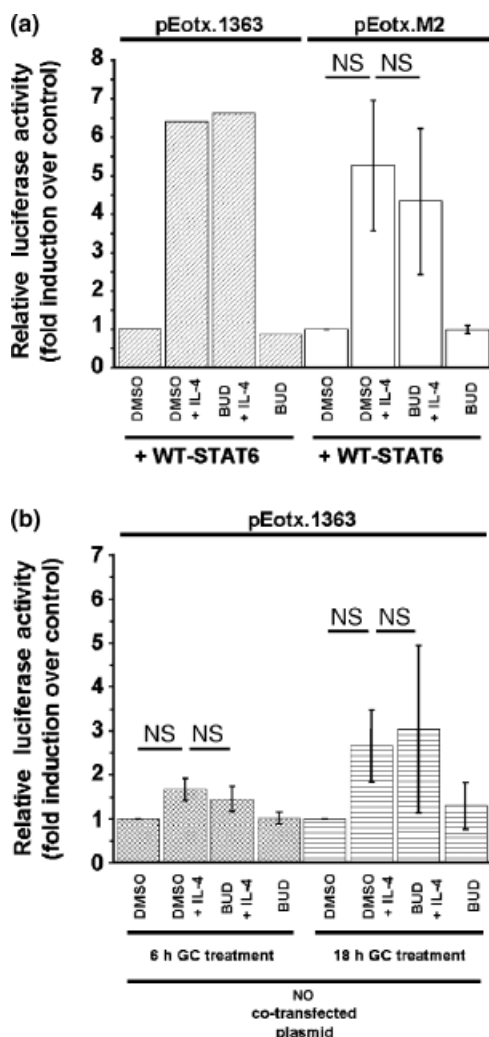


Fig. 5. Budesonide (BUD) did not inhibit signal transducer and activator of transcription (STAT)6-mediated transcription from the pEotx.1363 STAT6-driven reporter plasmid or the pEotx.M2 (pEotx.1363 with the mutated nuclear factor κ B (NF- κ B) site) STAT6-driven reporter plasmid, both derived from the eotaxin-1 promoter. (a) BEAS-2B were transfected with pEotx.1363 (left, striped bars, $n = 1$) or pEotx.M2 (right, open bars, $n = 3$) plus the WT-STAT6 expression vector overnight, and then treated as described in Fig. 4. Luciferase activity was calculated as described in Fig. 4. (b) BEAS-2B were transfected with pEotx.1363 alone and were treated as described in Fig. 4 with a 6 h BUD treatment (left, hatched bars) or an 18 h BUD pre-treatment protocol (right, horizontal striped bars). Values shown are means $\pm$ SEM of three independent experiments. NS ($P > 0.05$).

either reporter construct by IL-4 when empty pcDNA3 vector control was used in place of the WT-STAT6 expression vector (data not shown).

To be certain that endogenous STAT6 failed to respond to GC in a manner similar to that observed with co-transfected STAT6, induction of luciferase activity from the pEotx.1363 plasmid was also tested without any co-transfected expression plasmids using both a 6 and 18 h BUD pre-treatment. This experiment was similar to the pEOTAX/Luc-1363 transfection experiment carried out by Stellato et al. [41]. The pattern of luciferase induction was the same as reported previously, i.e. there was a small induction of luciferase activity by IL-4 and there was no significant BUD inhibition of the IL-4-induced luciferase activity after either a 6 h or the longer 18 h GC pre-treatment (Fig. 5b).

Discussion

The activation of STAT6 by IL-4 and IL-13 in airway epithelial cells is crucial for the development and maintenance of the allergic inflammatory response characteristically observed in asthma. STAT6 activates a host of inflammatory genes in these cells, including chemokines, IL-4, IL-13, VCAM-1, receptors for IgE and genes involved in goblet cell differentiation and mucus production. The expression of all of these genes and the associated responses are inhibited by GC [1, 3, 41, 42, 50, 53, 58]. Therefore, we hypothesized that STAT6 may be an important target of the anti-allergic action of GC in human airway epithelial cells. By using western blotting techniques and transfection of STAT6-driven reporter constructs, we observed that there was no effect of GC on the total cellular levels of STAT6 or phospho-STAT6 or on the appearance of either form of the protein in the nucleus. GC also had no effect on IL-4-induced STAT6-driven transcription in airway epithelial cells. We therefore conclude that GC are acting to suppress epithelial responses during allergic inflammation independent of the STAT6 pathway.

The analyses of the STAT6/phospho-STAT6 protein levels in the different cellular compartments were necessary to distinguish between potential GC effects on overall cellular levels of the STAT6 protein and any GC effects on STAT6 nuclear translocation and phosphorylation. Since there was no effect of GC on the total cellular levels of STAT6 protein, the results suggest that treatment with GC up to 24 h has no effect on expression of the STAT6 gene. GC are therefore unlikely to alter the generation or degradation rates of the STAT6 protein. Indeed, this observation of a lack of GC effect on total STAT6 protein levels parallels observations of a lack of GC effect on the stability of the STAT1 protein in peripheral blood mononuclear cells (PBMCs) [59]. While it is possible that longer incubations with GC (> 24 h) could alter either total or nuclear STAT6 or phospho-STAT6, based on the rapidity of the inhibitory effect of GC on eotaxin-1 and eotaxin-3 expression, we can eliminate this as a mechanism for inhibition of eotaxin-1 and eotaxin-3 expression in our experiments.

GC also did not inhibit the nuclear accumulation of STAT6 or phospho-STAT6 induced by IL-4. This suggests that GC do not affect the dimerization and movement of STAT6 to the nucleus, nor do they increase any potential nuclear phosphatase activity [60] since the levels of phospho-STAT6 remain unchanged in the presence of BUD. Although we did not specifically examine IL-4R α expression or early signal transduction events (e.g. JAK phosphorylation), the lack of effect of BUD on STAT6 nuclear translocation and/or phosphorylation argues against significant effects of GC on IL-4R α signalling in our experiments. Evidence from a recent report by Hu et al., examining GC effects on STAT1 signal transduction in response to IFN- γ , supports this hypothesis: there was no effect of DEX on the levels of cell surface IFN- γ R or Jak1 and Jak2 in PBMCs. Also, GC did not affect Jak1 activation as measured by Jak1 tyrosine phosphorylation.

Despite the minimal effect of GC on the levels of the activated form of STAT6 in the nucleus, we could not rule out the possibility that GC may have affected the ability of STAT6 to initiate transcription. For example, Biola et al. [48] have demonstrated that murine GR and STAT6 physically

interact with one another to antagonize transactivating function reciprocally in a mouse T lymphocyte cell line, and physical interactions between GR and STAT5 [46] and STAT3 [61] have also been reported. We therefore transiently transfected BEAS-2B cells with three different STAT6-driven reporter constructs, as well as a STAT6 expression vector, and monitored luciferase activity in response to GC treatment and IL-4 stimulation. As anticipated, based on the studies of STAT6/phospho-STAT6 protein, GC treatment failed to inhibit IL-4-stimulated luciferase reporter activity from all three STAT6-driven plasmids.

In the case of the mut-(C/EBP)-N₄-luc reporter plasmid, GC treatment led to slightly higher IL-4-induced luciferase reporter gene activity. GC enhancement of IL-4 signalling has been reported previously: it is known that GC augment IL-4-stimulated synthesis of IgE from mononuclear cells [62], probably via synergistic co-operation between STAT6 and C/EBP- β [55]. Since the C/EBP sites are mutated in the plasmid we used, which we verified by sequencing, the explanation of the observed enhancement in our experiments is currently unclear.

We co-transfected a vector expressing STAT6 in our studies in order to amplify the luciferase reporter signal in the BEAS-2B cells. The possibility that this additional STAT6 prevented a GC effect seems unlikely since we observed an identical luciferase response pattern when the empty pcDNA3 vector was transfected in place of the STAT6 expression vector but the magnitude of the induced luciferase activity was much reduced as expected. In addition, the amount of plasmid DNA expressing STAT6 used in the transfections was minimal: 20-fold lower than the amount of mut-(C/EBP)-N₄-luc reporter plasmid DNA. We have noted that the BEAS-2B and some other human immortalized airway epithelial cell lines appear to have lower endogenous STAT6 than primary human airway epithelial cells (N. M. Heller and R. P. Schleimer, unpublished results). In additional experiments, we found that GC failed to inhibit the appearance of nuclear-localized phospho-STAT6 after IL-4 stimulation using primary epithelial cells (data not shown). Therefore, it seems likely that our results using the BEAS-2B cell line can be readily extrapolated to primary epithelial cells, although formal demonstration of intact STAT6-driven transcription will require the ability to transfect these cells with high efficiency.

GC treatment did not significantly inhibit IL-4-induced luciferase reporter activity in the two eotaxin-1-derived reporter constructs. Interestingly, the general level of luciferase induction with the eotaxin-1-derived reporter constructs was about sixfold lower across all conditions when compared with the luciferase activities seen with the mut-(C/EBP)-N₄-luc reporter plasmid. This lower luciferase activity with the eotaxin-1-derived constructs is likely because of the presence of only a single STAT6 site in these constructs, compared with four STAT6 sites in the mut-(C/EBP)-N₄-luc construct. The synergistic effect of tandem STAT sites on transcriptional activity has been previously shown in the STAT1 [63], STAT3 [64], STAT4 [65, 66] and STAT5 systems [67, 68], although it has not been shown for STAT6.

Further confirmation of the negligible effect of GC treatment on STAT6 function was obtained from electrophoretic mobility shift assays data: GC treatment did not diminish the ability of STAT6 from BEAS-2B nuclear extracts to bind to a

STAT6 consensus sequence in an oligonucleotide probe (S. Matsukura, unpublished results). This observation contrasts with the inhibitory effect of 10^{-6} M DEX on the DNA binding capability of STAT1 from PBMCs [59].

The ability of GC to regulate IL-4R signalling and STAT6 activation is controversial. To our knowledge, there are two studies that suggest that GC can affect STAT6 signalling. The first suggesting an interaction between the GC and IL-4 signalling pathways, has been described in murine T lymphocytes [48]. In this study, IL-4 suppressed GC induction of the mouse mammary tumour virus promoter and GC suppressed transcription from a STAT6-driven promoter. Also, a physical interaction between murine GR and STAT6 proteins was observed. Our experimental system is quite different from this study: they used a murine cell line of haematopoietic origin and DEX treatment, while we used a human non-haematopoietic line and BUD treatment. As Biola et al. point out in their paper, 'the effect of GC on STAT6 transcriptional activity may (thus) depend on the promoter context, and also probably on the cell type.' Furthermore, concentrations of IL-4 (5 ng/mL) at which there is no inhibition of STAT6-dependent luciferase activity by GC are used to show physical association between STAT6 and GR in the co-immunoprecipitation experiments. Murine and human GR differ slightly in primary amino acid sequence, and species differences in function have also been shown [69]. This may explain the difference in the ability of GC to affect STAT6 function. For example, in contrast to the inhibition of murine T lymphocyte STAT6 function by DEX documented by Biola et al., one study of the effect of GC on *human* STAT6 found that human T lymphocyte STAT6 phosphorylation is *not* inhibited by DEX treatment [47]. In the second study, So et al. [70] showed that GC inhibited both IL-4-induced STAT6 phosphorylation and subsequent CD23 expression in both mononuclear and B cells, although supraphysiological concentrations (1–100 μ M) of GC were used in this study. Furthermore, GC do not inhibit IL-4-induced IgE production in B cells (a STAT6-dependent response) but rather they increase its synthesis [62]. In a recent study, Kibe et al. [71] showed that GC have no effect on airway epithelial responses to IL-13, such as MUC5AC expression, AHR and goblet cell hyperplasia in a mouse model. The reasons for the differences among these studies are unclear at present but may be because of the different cell types or activation conditions examined. However, our results clearly demonstrate that GC at pharmacologically relevant concentrations fail to inhibit IL-4R/JAK/STAT6 signalling in airway epithelium.

We speculate that GC may be blocking IL-4-induced gene expression downstream of STAT6 activation and function, for example, post-transcriptionally, translationally or post-translationally. Evidence from Stellato et al. [41] showed that eotaxin-1 mRNA decayed more quickly in the presence of BUD after transcription was inhibited with actinomycin D, suggesting that GC-mediated post-transcriptional mechanisms were in place to enhance the instability of the eotaxin-1 message. There are multiple genes that have been shown to be regulated post-transcriptionally by GC, including granulocyte macrophage-colony stimulating factor [72], COX2 [73], IL-1 [74] and IL-6 [75]. The mechanisms by which GC mediate post-transcriptional control of mRNA half-life may include inducing the expression or activity of an mRNA-destabilizing

protein such as tristetraprolin or AUF1 or conversely, GC may be inhibiting an mRNA-stabilizing protein such as HuR; both of these possibilities would lead to enhanced decay of the mRNA for eotaxin-1 [76]. It is also possible that GC are affecting the expression of IL-4-induced genes in epithelial cells at the level of protein translation or post-translationally, as is the case for IL-4R α [77].

In conclusion, STAT6 is unlikely to be a target of the anti-inflammatory action of GC, despite the potent inhibition by GC of expression of several important STAT6-driven genes in airway epithelial cells, including the eotaxins, MCP-4, TARC and MUC5AC [41, 78, 79]. We report here that despite the transrepressive effects of GC on these genes, GC have no inhibitory effect on the levels or function of the STAT6 transcription factor in human airway epithelial cells. This finding implies that GC inhibition of STAT6-driven genes may occur downstream of STAT6-dependent transcription, i.e. predominantly post-transcriptionally as appears to be the case for eotaxin-1 [41] and possibly also eotaxin-3. Further elucidation of the regulation of STAT6-induced genes by GC in allergic inflammation will be vital in better understanding the process by which GC prevent activation of epithelium in allergic diseases.

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