

ORIGINAL ARTICLE

Induction of glucocorticoid receptor- β expression in epithelial cells of asthmatic airways by T-helper type 17 cytokines

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Clinical & Experimental Allergy

Summary

Background Corticosteroid insensitivity in asthmatics is associated with an increased expression of glucocorticoid receptor- β (GR- β) in many cell types. T-helper type 17 (Th17) cytokine (IL-17A and F) expressions increase in mild and in difficult-to-treat asthma. We hypothesize that IL-17A and F cytokines alone or in combination, induce the expression of GR- β in bronchial epithelial cells.

Objectives To confirm the expression of the GR- β and IL-17 cytokines in the airways of normal subjects and mild asthmatics and to examine the effect of cytokines IL-17A and F on the expression of GR- β in bronchial epithelial cells obtained from normal subjects and asthmatic patients.

Methods The expression of IL-17A and F, GR- α and GR- β was analysed in bronchial biopsies from mild asthmatics and normal subjects by Q-RT-PCR. Immunohistochemistry for IL-17 and GR- β was performed in bronchial biopsies from normal and asthmatic subjects. The expression of IL-6 in response to IL-17A and F and dexamethasone was determined by Q-RT-PCR using primary airway epithelial cells from normal and asthmatic subjects.

Results We detected significantly higher levels of IL-17A mRNA expression in the bronchial biopsies from mild asthmatics, compared with normal. GR- α expression was significantly lower in the biopsies from asthmatics compared with controls. The expression of IL-17F and GR- β in biopsies from asthmatics was not significantly different from that of controls. Using primary epithelial cells isolated from normal subjects and asthmatics, we found an increased expression of GR- β in response to IL-17A and F in the cells from asthmatics ($P \leq 0.05$). This effect was only partially significant in the normal cells. Dexamethasone significantly decreased the IL-17-induced IL-6 expression in cells from normal individuals but not in those from asthmatics ($P \leq 0.05$).

Conclusion Evidence of an increased GR- β expression in epithelial cells following IL-17 stimulation suggests a possible role for Th17-associated cytokines in the mechanism of steroid hypo-responsiveness in asthmatic subjects.

Keywords dexamethasone, glucocorticoid receptor, IL-17A, IL-17F, mild asthma, primary airway epithelial cells, steroid insensitivity, Th17 cells

Submitted 15 January 2010; revised 18 April 2010; accepted 27 April 2010

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Introduction

Asthma is a common chronic inflammatory disease of the lung [1]. Although the majority of asthmatics have mild to moderate disease, between 10% and 15% of patients suffer from severe, difficult to treat asthma and represent considerable management challenges [2]. A characteristic of

severe asthmatics is that they are relatively unresponsive to treatment including high doses of inhaled and systemic corticosteroids [3, 4]. Several mechanisms have been proposed to explain the causes of poor response to steroids including the up-regulation of the glucocorticoid receptor- β (GR- β) [5–8]. GR- β has been shown to act as a

dominant negative inhibitor of GR- α , which is the active isoform of this receptor [9]. Overexpression of GR- β in several transfected cell lines (COS-1, CV-1 and COS-7) inhibited the trans-repression activity of GR- α on the activities of AP-1 and NF- κ B [10, 11]. However, the generally lower relative abundance of GR- β compared with that of GR- α and its physiological significance are currently under debate [12–18].

We and others have provided evidence that the GR- β expression increases in asthmatic subjects. Leung et al. [19] and Hamid et al. [20] showed that peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage cells from glucocorticoid-insensitive asthmatics contained greater numbers of GR- β -positive cells compared with glucocorticoid-sensitive subjects. Using immunocytochemistry, we recently showed that there is also an up-regulation of GR- β in biopsies of severe asthmatics compared with well-controlled patients [21]. Interestingly, we have demonstrated that the expression of GR- β is not only restricted to inflammatory cells but also that epithelial cells express a considerable level of GR- β immunoreactivity [21].

Type I steroid insensitivity is a property acquired by some cells that are chronically stimulated by a number of pro-inflammatory factors including allergens, microbial super-antigens and several cytokines (e.g. IL-2, IL-4, TNF- α and IFN- γ) [3, 22, 23]. In particular, cytokines IL-2 and IL-4 up-regulate GR- β expression in peripheral blood T lymphocytes, thus inducing corticosteroid resistance [19]. Severe asthma is characterized by an increased infiltration of both neutrophils and eosinophils [24] and does not exhibit the classical T-helper type 2 (Th2) cytokine profile that is observed in mild disease [25, 26]. Although acquired steroid resistance has been extensively studied in severe or unstable asthma patients [3, 12, 19, 27], further investigation of this phenomenon on the milder asthmatics is of interest.

The cells of the epithelial layer in these patients are exposed to many inflammatory stimulants during exacerbations, in similarity to severe asthma, and are consequently induced to produce diverse pro-inflammatory substances and soluble pro-fibrotic mediators, which contribute to the enlargement of airways' wall thickness and bronchoconstriction in asthmatics [28]. It has been suggested that the epithelial cells from asthmatics could behave biochemically and physiologically differently than those from normal subjects [29–31]. In asthmatics, histopathologic evidence has revealed important alterations of the airway epithelial cell layer [28, 32]. Nevertheless, the impact of these alterations on the epithelial cell's responsiveness to steroid therapy requires further investigation.

We recently reported an increased infiltration of Th17 lymphocytes and a concomitant mRNA expression of both IL-17A and F mRNA expression in bronchial biopsies from

mild and severe asthmatics [33]. Therefore, we hypothesized that IL-17 cytokines can induce the expression of GR- β in the airway epithelial cells from mild asthmatics, which in turn may contribute to acquired steroid insensitivity. In this study, our first aim was to confirm the expression of the GR- β and IL-17-associated cytokines in the airways of normal subjects and mild asthmatic patients. We then examined the effects of cytokines IL-17A and F on the expression of GR- β and GR- α , and their potential impact on the steroid hypo-responsiveness of primary airway epithelial cells from normal and asthmatics.

Methods

Tissues from normal subjects and mild asthmatics

Bronchial biopsies were obtained from healthy non-smoking adult (29 ± 19.3 years old) individuals ($n = 20$) with no asthma history and not taking any medication. Fifteen mild asthmatic patients (30 ± 9.6 years old) were studied. The demographic and spirometry data are presented in Table 1. Nine of 10 of the asthmatic patients were taking a β_2 -adrenergic receptor agonist (Salbutamol; Fenoterol; Terbutaline) and one in four patients were taking a steroid (Budesonide; Fluticasone; Emocort). Bronchoscopy was performed on adult subjects recruited at the Montreal Chest Institute of the McGill University Health Centre who signed an informed consent form, following the directions of the approved protocol by the

Table 1. Demographic characteristics of normal and mild asthmatic subjects and spirometry data

Parameters	Normal	Mild asthmatics	<i>P</i> -values
Sex ratio male/female	1.0	1.2	
Age (median)	29 ± 19.3	30 ± 9.6	0.2847
Ethnicity	Caucasians	Caucasians	
Body mass index	30.1 ± 10.1	27.1 ± 7.3	0.4219
Disease since	N/A	1991.3 ± 5.6	
FEV ₁ (L) (predicted)	3.8 ± 1.1	3.3 ± 0.8	0.204
FEV ₁ % predicted	103.9 ± 15.7	90.2 ± 14.7	0.0069**
FVC (L) (predicted)	4.6 ± 1.2	4.4 ± 1.1	0.3969
FVC % predicted	105.2 ± 12.3	100.3 ± 14.9	0.1479
FEV ₁ /FVC (%)	81.3 ± 6.4	76.0 ± 9.7	0.0173**
Atopy (%)	44.0	90.0	
*Patients with medication (%)	No	β_2 -agonists: 88% of patients Steroids: 28% Both medications: 17%	

* β_2 -agonists: Salbutamol; Fenoterol; Terbutaline.

**Medians significantly different ($P \leq 0.05$). Wilcoxon's non-parametric signed-rank test.

Steroids: Budesonide; Fluticasone; Emocort.

FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

Ethics Committee of the hospital. Bronchial samples were immediately placed in RNAlater (Ambion, Austin, TX, USA) and frozen at -80°C until further use.

Cell culture and treatments

Primary airway bronchial epithelial cells isolated from normal and asthmatic subjects were used because they are in general presumably a better experimental model than immortalized epithelial cell lines [34, 35], which may or may not represent adequately the behaviour and/or phenotype of airway epithelial cells from asthmatics. The primary bronchial epithelial cells (PBECs) were isolated using techniques described previously [36]. These cells have been characterized for the expression of cytokeratin by immunofluorescence and flow cytometry [37]. Selected asthmatic donors [mean forced expiratory volume in 1 s (FEV_1) = $85 \pm 3.1\%$ and mean PC_{20} = 4.0 ± 2.2 mg/mL] were atopic, non-smokers and were not taking inhaled or systemic corticosteroids but used inhaled β_2 -agonist (Salbutamol p.r.n.) on demand. Normal subjects (mean FEV_1 = $98.2 \pm 5.1\%$ and mean PC_{20} = 99.4 ± 1.2 mg/mL) were non-atopic, non-smokers with no history of asthma or systemic diseases. PBECs at passage 4 were used. The cells were cultured in complete medium [Dulbecco's modified Eagle's medium (DMEM)+Ham's F12 at 3:1 proportion and supplemented with: 10 ng/mL human epidermal growth factor (Austral Biologicals, San Ramon, CA, USA)], 24.3 $\mu\text{g/mL}$ adenine, 5 $\mu\text{g/mL}$ crystallized bovine insulin, 5 $\mu\text{g/mL}$ human transferrin, 2×10^{-9} M 3,3',5',triiodo-L-thyronin (Sigma Chemicals, St Louis, MO, USA), 0.4 $\mu\text{g/mL}$ hydrocortisone (Calbiochem, EMD Chemicals, Gibbstown, NJ, USA), 10^{-10} M cholera toxin (Sigma Chemicals), 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin G and streptomycin, and 25 $\mu\text{g/mL}$ gentamicin (Sigma Chemicals) [37]. The cells were plated in complete medium at a density of 5×10^5 cells/mL in triplicate. On the following day, cells were starved for 24 h in basal medium (DMEM+F12 without supplements). The cells were stimulated with recombinant human IL-17A and F cytokines (50 ng/mL; R&D, Minneapolis, MN, USA) either alone or combined in fresh starving medium, during 0, 3, 6 and 24 h and then harvested for RNA extraction. To determine the cells' response to dexamethasone, PBECs (normal and asthmatics) were starved 24 h and then stimulated with a combination of IL-17A and F for 16 h to induce GR- β expression, because preliminary experiments indicated that GR- β expression peaked between 6 and 24 h of cytokine stimulation. Our hypothesis is that these cytokines induce GR- β expression, which then would antagonize the function of GR- α , according to the proposed mechanism [9, 10]. This approach would ensure that upon addition of steroid, GR- β levels are higher than those of non-stimulated controls, so that it could be associated with a reduced response to steroid. After stimulation, the medium was

replaced with fresh starving medium containing both cytokines and dexamethasone. Preliminary experiments with different doses of corticosteroids were used to determine that 1 μM provided the best response. The cells and supernatants were harvested at 0, 3, 6 and 24 h post-dexamethasone treatment.

RNA extraction and real-time reverse transcription polymerase chain reaction

Extraction of total RNA from bronchial biopsies and from cultured epithelial cells was performed with the RNeasy Mini kit (Qiagen, Mississauga, ON, Canada), as directed by the manufacturer's instructions. DNase I treatment was done before the elution of the RNA from the column. Quality control and RNA concentration were determined using the Experion electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA). The reverse transcription reaction (40 μL) contained 1 μg of total RNA, 1 μL of reverse transcriptase Superscript II (Invitrogen), 0.5 μL of RNase inhibitor, 8 μL 5 $\times$ first strand buffer (Invitrogen), 4 μL of 0.1 M DTT, 0.5 mM of each dNTP and 0.5 μg of oligo dT12-18 primers (Invitrogen). RT was carried out at 42°C for 1 h, followed by 93°C for 5 min and then rapid cooling to 4°C . Real time PCR amplification of cDNA targets was performed using a StepOnePlus Real Time thermal cycler and Power SYBR Green PCR Kit (both from Applied Biosystems Inc., Foster City, CA, USA), which contains the dye Rox, a passive reference used to normalize the signal from SYBR Green/dsDNA and to correct for well-to-well fluorescent fluctuations. The following forward and reverse primers were used: GR- β (forward 5'-GAA GGA AAC TCC AGC CAG AAC and reverse 5'-TGA CTT ATT ATT GAC AAC GAA GTG C); GR- α (forward 5'-GAA GGA AAC TCC AGC CAG AAC and reverse 5'-CTG ATT GGT GAT GAT TTC AGC TA); IL-17A (forward 5'-CAT CCA TAA CCG GAA TAC CAA TA and reverse 5'-TAG TCC ACG TTC CCA TCA GC); IL-17F (forward 5'-GTG CCA GGA GGT AGT ATG AAG C and reverse 5'-ATG TCT TCC TTT CCT TGA GCA TT); IL-6 (forward 5'-ACC TTC CAA AGA TGG CTG AAA and reverse 5'-GCT CTG GCT TGT TCC TCA CTA C); GAPDH (forward 5'-AGT CAA CGG ATT TGG TCG TAT T and reverse 5'-ATG GGT GGA ATC ATA TTG GAA C). The PCR reactions were carried out in Fast Optical 96-well Reaction plates (Applied Biosystems). For all wells, each reaction (10 μL) contained: 5 μL of 2 $\times$ Power SYBR Green PCR master mix (Applied Biosystems), 0.5 μM each forward and reverse primers, 2 μL of cDNA template (equivalent to 50 ng cDNA) and certified DNA-free water (Medicorp, Montreal, QC, Canada). The cycling conditions were: 95°C for 10 min followed by 40 cycles of 95°C for 20 s, 60°C for 20 s and 72°C for 30 s. At the end of the amplification, a melting temperature analysis of the amplified gene products was performed routinely for all cases: the PCR products were melted by gradually

increasing the temperature from 60 °C to 95 °C in 0.3 °C steps, and the dissociation curves were generated with the Melting Curve analysis tool of the StepOne software (Applied Biosystems). We confirmed that only one product was consistently amplified in all PCR reactions. The negative water and –RT controls showed no amplification. The sizes of PCR gene products were verified by agarose gel electrophoresis stained with ethidium bromide: GR-β, 204 bp; GR-α, 169 bp; IL-17A, 174 bp; IL-17F, 200 bp; IL-6, 154 bp; and GAPDH, 139 bp. The amplification efficiencies of GAPDH, GR-α and GR-β primer sets were determined from standard curves (dilution series 1 : 1, 1 : 10, 1 : 100, 1 : 1000 and 1 : 10 000), with the relative concentrations expressed in arbitrary units. The relative standard curve method was used to determine the relative expression levels of GR-α and GR-β [38]. In this method, the amplification efficiency of each gene is estimated from the standard curve. The StepOne software interpolates the C_t values of both the controls and asthmatics to the standard curves. The results from the controls and asthmatics are then normalized by dividing the target gene (e.g. GR-β) result with their corresponding endogenous control result (GAPDH). This is mathematically represented by a formula derived from the $\Delta\Delta C_t$ method [38, 39]:

$$NGE = (E_{\text{target}})^{-C_{t\text{target}}} / (E_{\text{ref}})^{-C_{t\text{ref}}}$$

where NGE is the normalized quantity of target gene divided by the quantity of endogenous gene control; E_{target} and E_{ref} are the amplification efficiencies of the target and reference genes, respectively, which are determined from standard curves; and $C_{t\text{target}}$ and $C_{t\text{ref}}$ are the number of amplification cycles at the threshold set for the target and reference genes, respectively.

Western immunoblot

Western immunoblots were performed following standard techniques. Briefly, epithelial cells from normal and asthmatics were cultured in medium with or without IL-17A and F combined for 24 h. Whole cell lysates were prepared in RIPA buffer [50 mM Tris, 150 mM NaCl, 0.5% SDS, 1% Triton X-100 and protease inhibitors cocktail (Roche Applied Science, Indianapolis, IN, USA)]. Extracts were clarified at 14 000 g at 4 °C for 10 min and protein concentrations were determined using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA). Forty microgrammes of total protein extracts and a Precision Plus Protein Kaleidoscope Standard (Bio-Rad) were separated on denaturing 7.5% SDS-polyacrylamide gels, electro-blotted into nitrocellulose membranes (Bio-Rad) and probed with rabbit anti-human GR-β (Abcam, Cambridge, MA, USA) overnight at 4 °C, or with mouse anti-human GAPDH antibody (Affinity Bioreagents, Thermo Scientific,

Rockford, IL, USA) to verify equal loading among the samples. After wash, secondary goat anti-rabbit IgG conjugated to Dylight800 (Thermo Scientific) or goat anti-mouse IgG conjugated to Dylight680 was added and the membranes were scanned using the Odyssey Infra Red Imager (LI-COR Biosciences, Lincoln, NE, USA). Densitometry analysis of the bands was performed with the GIMP software (GNU Image Manipulation Program; <http://www.gimp.org>); the integrated optical density of each signal band was obtained with a histogram on a grey scale ranging from 0 (black) to 255 (white), and expressed as the ratio of 1 to the mean pixel intensity corrected for background.

Immunostaining and confocal microscopy

Immunocytochemistry for IL-17 and GR-β on sections from bronchial biopsies was performed as described previously [21, 33]. For immunostaining of primary epithelial cells, they were cultured in sterile glass cover-slides coated with poly-lysine in medium as described above. The cells were stimulated with IL-17 cytokines (50 ng/mL) or non-stimulated for 16 h, followed by the addition of dexamethasone (1 μM) for 45 min and then the cells were fixed with 4% paraformaldehyde in PBS for 15 min. After permeabilization with PBS+0.1% Triton X-100, the cells were blocked (Dako Canada, Mississauga, ON, Canada) and stained with rabbit anti-GR-α or rabbit anti-GR-β specific antibodies (Abcam). Secondary anti-rabbit-Alexa 488-conjugated antibody was then added and specimens were visualized using a Zeiss confocal microscope (Carl Zeiss Canada Ltd, Toronto, ON, Canada).

Statistics analysis

The values were analysed by one-way ANOVA to determine if significant differences existed among the cell populations and/or treatments. When significant differences were found ($P \leq 0.05$), Tukey's, Student's or Wilcoxon's non-parametric tests were performed using the Prism 5 software (GraphPad Software, La Jolla, CA, USA).

Results

Interleukin-17A and F and glucocorticoid receptor-β expression in bronchial biopsies

As a first approach, we examined the mRNA expression of IL-17A and F in bronchial biopsies from normal and mild asthmatics. We demonstrated the expression of both IL-17A and F in all biopsies (Figs 1a and b). The expression of IL-17A mRNA was significantly higher in the mild asthmatics compared with normal subjects ($P \leq 0.05$). The expression of IL-17F mRNA in the asthmatics was not significantly different to that of normal subjects. GR-α mRNA levels were significantly lower in the mild

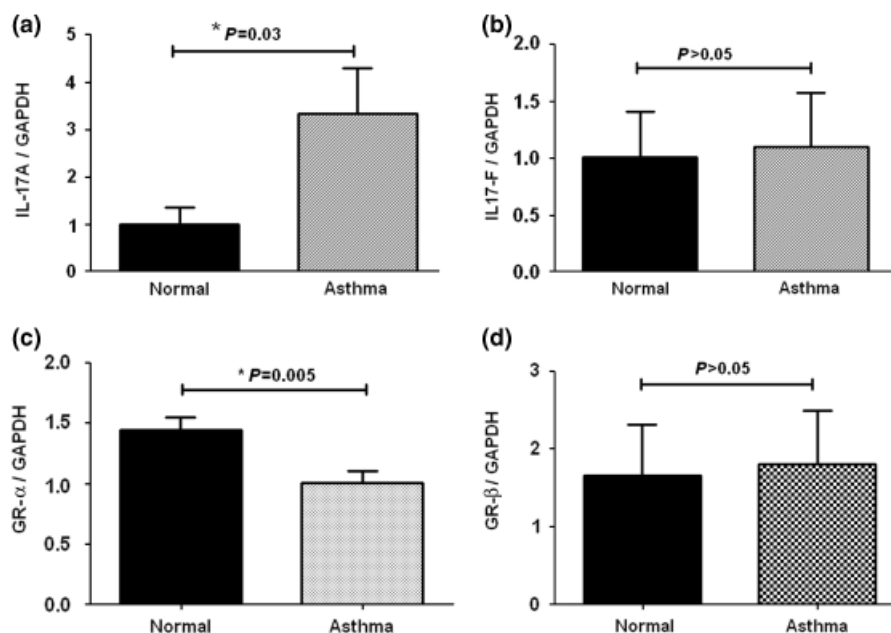


Fig. 1. IL-17A (a) and IL-17F (b) mRNA relative expression levels in bronchial biopsies from normal ($n = 12$) and from mild asthmatic patients ($n = 11$). IL-17A and F mRNA were quantitated by real-time RT-PCR. (c) Glucocorticoid receptor (GR)- α and (d) GR- β mRNA relative expression in the same biopsies from normal and asthmatic subjects. Gene expression was normalized with GAPDH. *Significant differences of the means were determined by Student's T -test ($P < 0.05$).

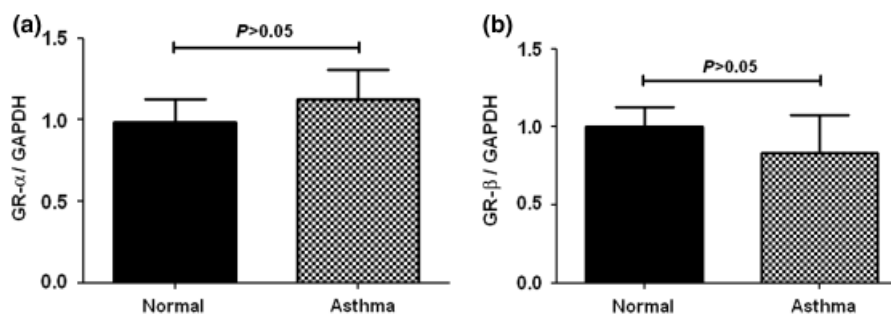


Fig. 2. Glucocorticoid receptor (GR)- α (a) and GR- β (b) mRNA relative expression in primary bronchial epithelial cells (PBEs) from normal ($n = 5$) and from asthmatic individuals ($n = 4$) in complete culture medium without cytokine stimulation. GR- α and GR- β mRNA were quantitated by real-time RT-PCR and the results are expressed relative to the normal cells. No significant differences were observed ($P > 0.05$).

asthmatics compared with the normal subjects (Fig. 1c). GR- β expression was not significantly different in the mild asthmatics compared with the normal subjects (Fig. 1d).

Glucocorticoid receptor- β mRNA is increased by interleukin-17A and F in vitro

To determine the effect of exogenous IL-17A and F on GR- β expression, we used PBEs from normal subjects and mild asthmatics. Under basal conditions, epithelial cells from both groups express similar levels of GR- α and GR- β mRNA (Figs 2a and b). The average relative abundance (basal) of GR- β mRNA was about 1500-fold

lower than that of GR- α . Stimulation with IL-17A and F, either alone or combined, had no significant effect on GR- α mRNA levels in the primary epithelial cells from normal and asthmatics (Figs 3a and b). When epithelial cells from normal subjects were stimulated with IL-17A or F either alone or in combination, the expression of GR- β mRNA increased significantly higher than the basal expression only at 6 h of post-stimulation with IL-17A alone and significantly higher at 6 and 24 h of post-stimulation with IL-17F alone (Fig. 3c). In the asthmatic epithelial cells, GR- β expression increased significantly over the basal expression at 3, 6 and 24 h of post-stimulation with IL-17F alone and with IL-17A and F combined (Fig. 3d). Even though

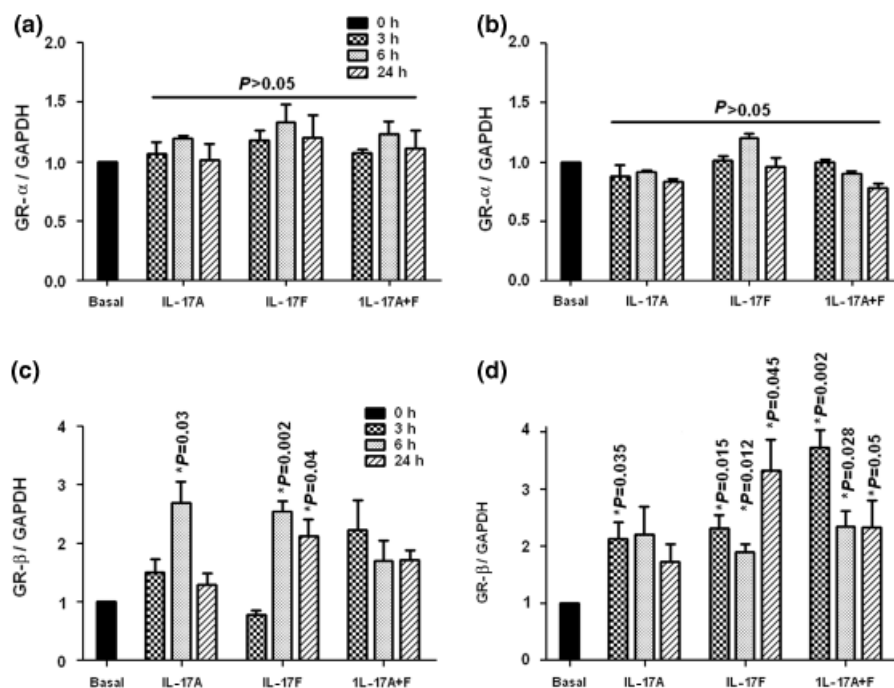


Fig. 3. Effect of cytokines IL-17A and F on glucocorticoid receptor (GR)- α (a and b) and GR- β (c and d) mRNA expression in primary airway epithelial cells from normal and asthmatic patients. The cells were starved for 24 h, then stimulated with IL-17A and F either alone or combined, during 3, 6 and 24 h. The cells were harvested and total RNA was extracted followed by reverse transcription and quantitative PCR. GR- α and - β expression values were normalized with GAPDH. The black-filled bars indicate basal expression (0 h); bars with black-and-white squares indicate stimulation for 3 h; bars with black dots indicate stimulation for 6 h; bars with diagonal lines indicate stimulation for 24 h. *Significance was determined by ANOVA and Tukey's multiple comparisons test ($P \leq 0.05$).

both normal and asthmatic epithelial cells responded to IL-17 stimulation, the asthmatic cells responded better as indicated by a greater number of time-points where GR- β expression was statistically above the basal expression.

Dexamethasone suppressed interleukin-17-induced interleukin-6 mRNA expression in primary bronchial epithelial cells from normal but not from asthmatic subjects

To determine if the changes in GR- β expression in epithelial cells are associated with a decreased steroid responsiveness, we stimulated the PBECs from normal and asthmatic subjects with IL-17A and F combined for 16 h, a period enough to increase both GR- β and IL-6 expression, followed by the addition of dexamethasone. We then determined the extent of inhibition of IL-6 mRNA expression (induced by IL-17A and F) in response to the steroid when compared with basal control. IL-6 expression significantly increased twofold and 1.5-fold in the asthmatic and normal cells, respectively, as compared with the non-stimulated cells (0 h) (Figs 4a and b). This indicates that the response of both cell types to IL-17A and F cytokines stimulation was statistically similar. Furthermore, IL-6 expression was significantly inhibited by dexamethasone

in the epithelial cells (in both non-stimulated and stimulated) from normal subjects at 3, 6 and 24 h of steroid addition (Fig. 4a). Dexamethasone also decreased the IL-17-induced IL-6 expression at all time-course points analysed in the primary epithelial cells from asthmatics, but such an effect failed to be significantly different from the basal controls (0 h) ($P \leq 0.05$) (Fig. 4b).

Expression of glucocorticoid receptor- β protein in epithelial cells

Using confocal microscopy, we confirmed that dexamethasone-induced GR- α translocation to the nucleus in both control (non-stimulated) and IL-17-stimulated epithelial cells. An increased immunoreactivity for GR- β in the stimulated cells compared with non-stimulated cells was also observed. The staining of GR- β was mainly perinuclear, although nuclear localization was also observed (Fig. 5a). To provide quantitative data of the GR- β protein, we performed Western immunoblots with these cells (Fig. 5b). Stimulation with cytokines IL-17A and F did not significantly increase GR- β protein in both normal and asthmatics when compared with control non-stimulated cells. Nevertheless, taken as a whole, the asthmatic cells expressed significantly higher GR- β protein levels than the normal (Fig. 5c).

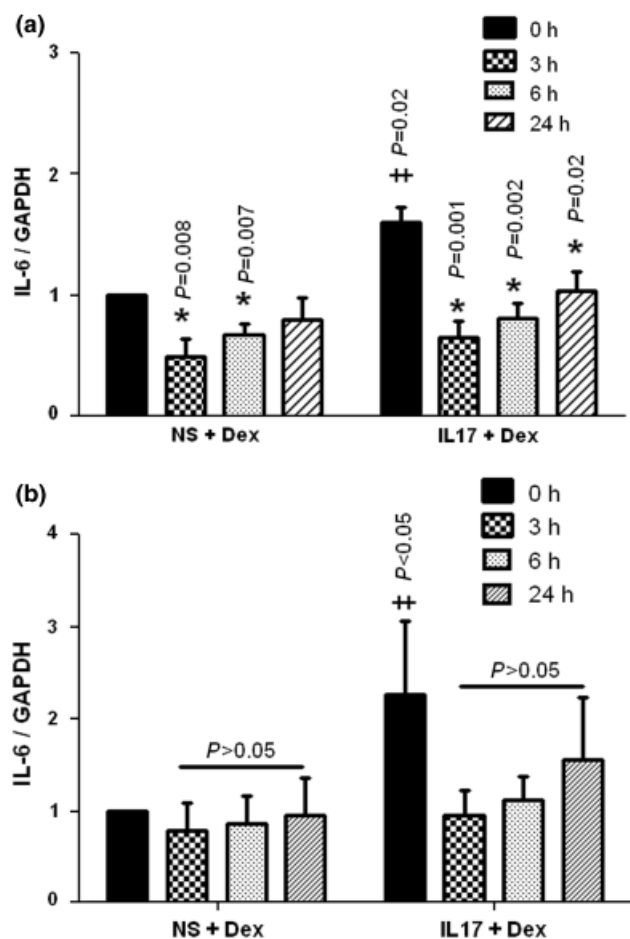


Fig. 4. Dexamethasone significantly suppressed IL-17-induced IL-6 mRNA expression in primary airway epithelial cells from normal (a) individuals but not in those from mild asthmatic (b) subjects ($n = 5$). The cells were either non-stimulated (NS) or stimulated with IL-17A + F for 16 h to induce GR- β and IL-6, followed by the addition of dexamethasone and the culture continued for 3, 6 and 24 h. The IL-6 mRNA expression was determined by real-time RT-PCR, normalized with GAPDH. *Significantly different ($P < 0.05$) when compared with the corresponding time-point 0 h of each group. †Significantly different to time-point 0 h (NS + Dex). Two-way ANOVA and Tukey's multiple comparison tests were used to determine significant differences of the means.

Discussion

We have investigated the relationship between Th17-associated cytokines and the expression of GR- β , which is one of the possible mechanisms for glucocorticoid insensitivity in asthma. Several lines of evidence suggest a role for GR- β expression. This variant of the GR family is also expressed in human lung epithelial cells and has been demonstrated to be up-regulated in steroid non-responsive asthmatics [19–21]. In addition, specific cytokines including IL-4, IL-2, TNF- α and IFN- γ can induce GR- β expression, resulting in acquired steroid hypo-responsiveness [12, 19, 23]. Recently, Th17 infiltration and asso-

ciated cytokine expression were demonstrated to be up-regulated in airway biopsies from asthmatic patients [33]. In the present study, we obtained data confirming that the IL-17A (but not IL-17F) mRNA expression was significantly higher in the biopsies from mild asthmatics than the normal subjects, although such an increase was not associated to a significant change in GR- β mRNA levels (Fig. 1). We further explored the relationship between Th17 cytokines and GR- β by examining the effect of IL-17A and F on the GR- β expression using primary epithelial cells from normal subjects and mild asthmatics. Although the asthmatic epithelial cell donors were not steroid hypo-responsive, this was not a critical constraint in testing our hypothesis. Our study focused on a possible mechanism for steroid hypo-responsiveness at the *in vitro* level, which could help to further understand the observed hypo-responsiveness in asthmatic patients.

Interestingly, we found that the GR- β mRNA can be induced by IL-17A and F, either alone or in combination, and that this induction was more significant in epithelial cells from asthmatics (Fig. 3). GR- β mRNA consistently increased significantly above the basal levels when the epithelial cells were stimulated with combined IL-17A and F at the three time-points analysed (3, 6, 24 h); IL-17F alone significantly induced GR- β at time point 3 and 6 h, whereas IL-17A alone induced GR- β significantly only at time-point 3 h (Fig. 3). This suggests that IL-17F alone could be more effective than IL-17A alone in inducing GR- β , or that the inducible effect of IL-17F and the combination of both cytokines persisted longer than that of IL-17A alone. The apparent discrepancy in the GR- β expression between the tissues (*in vivo*) and cells (*in vitro*) could be explained at least in part by the different physiological/biochemical conditions in these two systems. The increased levels (more than threefold in the asthmatic cells) are in good agreement with the results published by others. For example, Bergeron et al. [21] showed about a twofold increase in the number of GR- β immunoreactive epithelial cells of the airways, whereas Tliba et al. [23] reported that in airway smooth muscle cells stimulated with IFN- γ and TNF- α combined, GR- β mRNA increased up to 70% over the basal control.

We then examined whether the stimulation of PBECs with IL-17 cytokines leads to acquired dexamethasone insensitivity. We used the expression of IL-6 as a marker for steroid response in epithelial cells. This cytokine is widely expressed, has been reported to be increased in asthmatic subjects and is known to be mainly produced by epithelial cells [40, 41]. We found that following treatment with IL-17, dexamethasone significantly decreased the expression of IL-6 in cells from normal individuals but not in those from asthmatics. There were no differences in IL-17A receptors that could help to explain why the normal epithelial cells were significantly more sensitive to the steroid than the asthmatic epithelial cells.

Importantly, the asthmatic cells expressed significantly higher GR- β protein levels than the normal cells (Fig. 5). This could explain, at least in part, the lower steroid sensitivity of the asthmatic cells observed for the higher expression of IL-6 than in the normal cells in response to IL-17 cytokines. Therefore, the observed differences may reflect the phenotypic differences reported in epithelial cells from mild asthmatics compared with those from control subjects [37, 42]. Our results go along with that of

other reports suggesting that cells exposed to inflammatory agents *in vitro* could develop some degree of steroid insensitivity, known as steroid insensitivity type I [3, 22, 23, 43]. Hypothetically, it may be possible that repeated exacerbations and frequent stimulation over the years would induce biochemical and/or physiological modifications [29–31], which in turn could affect the cell's responsiveness to steroids. For instance, it is known that combined cytokines IL-2 and IL-4 can decrease the affinity of the GR for its ligand, which may lead to steroid hypo-responsiveness [44].

Alternatively to the GR- β mechanism, a decrease in GR- α levels could attenuate the anti-inflammatory actions of corticosteroids [18, 45]. The airway tissues from asthmatics had significantly lower GR- α mRNA levels than those from normal individuals (Fig. 1). However, due to the inevitable heterogeneity of the biopsies, we cannot assume that this result originates solely from the bronchial epithelium. To circumvent this ambiguity, we used primary epithelial cells in culture from normal and asthmatic individuals not taking glucocorticosteroids. No differences in GR- α were observed in epithelial cells from normal and asthmatic patients cultured without stimulation (Fig. 2). Furthermore, stimulation with IL-17A and F cytokines failed to significantly affect the GR- α expression *in vitro* (Fig. 3). Therefore, the decreased GR- α levels in the biopsies from asthmatics could not be associated to the increased IL-17 levels, at least not at the epithelial compartment; rather, a factor(s) other than IL-17A and F cytokines may have caused the reduction in GR- α levels in the asthmatic tissues. It is well known that GR- β and GR- α expression can be affected by a number of factors such as cytokines, allergens, super-antigens, hypoxia, prolonged glucocorticoid treatment, etc. [3, 16, 22, 23, 45–47].

Although the cells from asthmatics developed some degree of insensitivity to steroids after exposure with IL-17, the association between high expression of GR- β and

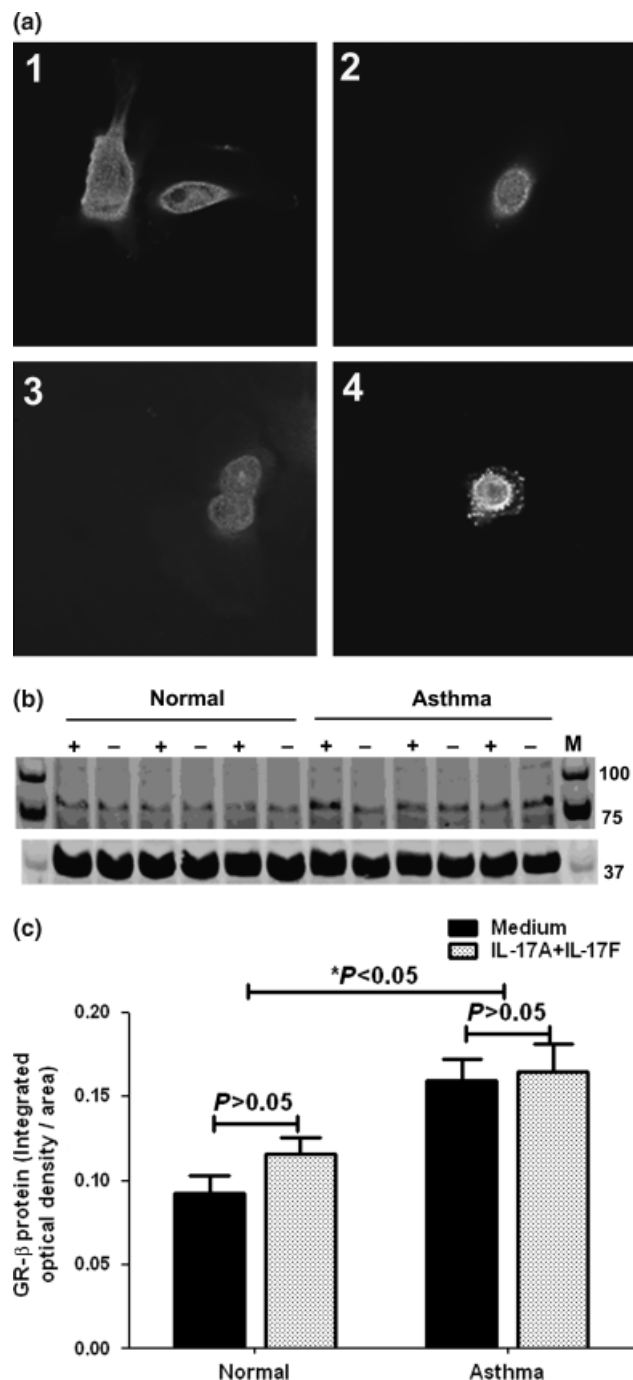


Fig. 5. Expression of glucocorticoid receptor (GR)- β protein in epithelial cells from normal and asthmatics. (a) Laser confocal immunofluorescence of GR- α (1 and 2) and GR- β (3 and 4) in primary airway epithelial cells. The cells were either non-stimulated (1 and 3) or stimulated with IL-17A+F (2 and 4) for 24 h followed by 1 μ M dexamethasone. The cells were fixed, permeabilized and immunolabelled with GR- α - or GR- β -specific antibodies. (b) GR- β protein expression detected by Western immunoblotting in whole cell lysates from *in vitro* cultured primary bronchial epithelial cells (PBEs) for 24 h as described in "Materials and methods". Anti-GAPDH antibody was used to verify equal protein loads. Symbols + and - refer to cells stimulated or not with combined IL-17A and F for 24 h. M is the protein ladder markers (kDa). This gel picture is representative of $n = 3$. (c) Densitometry analysis of immunoblottings performed with PBEs whole cell lysates collected after 24 h either stimulated or not with combined IL-17A and F. Two-way ANOVA and Tukey's multiple comparison tests were used to determine significant differences of the means. *Significant differences between normal and asthmatic epithelial cells ($P < 0.05$).

low sensitivity to dexamethasone may be either direct or indirect. It is important to note that a number of investigators have shown a disassociation of steroid insensitivity in cultured cells measured by cytokine release and the expression of GR- β [48, 49]. The relative low abundance of GR- β compared with GR- α and its significance in the development of glucocorticosteroid resistance in inflammatory diseases still remains a matter of controversy [12–14, 16, 17, 50, 51]. Human GR- β 's proposed function as a dominant negative inhibitor of GR- α activity has been demonstrated in transfected cells over-expressing GR- β protein; this mechanism involves the formation of transcriptionally impaired GR- α /GR- β heterodimers [9, 10]. It is speculated that, in order to have a significant effect on GR- α inhibition, GR- β protein should be at least as abundant as GR- α [51, 52]. Although the abundance of GR- β mRNA has been reported to be much lower than GR- α in several human tissues and cells, including PBMC, relative increases in GR- β mRNA or protein have been consistently observed in the context of inflammatory diseases, which may be associated with glucocorticoid resistance [16–18, 20, 52, 53]. In the immortalized human bronchial epithelial cell line BEAS 2B, Pujols et al. [47] reported that the relative abundance of GR- β was 2800-fold lower than the GR- α messenger, which is even lower than the levels detected in the primary epithelial cells from this study (about 1500-fold). Although IL-17A and F stimulation did not significantly increase GR- β protein in the primary epithelial cells, we found that the asthmatic cells expressed higher levels than the normal cells, and this is associated to the lower steroid response in the asthmatic cells. The contrasting results between mRNA and protein levels may result from the distinct accuracy and sensitivity of these techniques, and from the distinct dynamic mechanisms that regulate (turn-over) these molecules. Conflicting results on the quantification of GR- β protein have been reported, which could be explained by the different cells or tissues analysed, by the different antibodies used, by post-translational modifications of the protein affecting binding by the antibody and by the methodology used [50, 51]. Moreover, the hypothesis stating that GR- β acts as a dominant negative inhibitor of GR- α has been put in doubt, while alternative roles or functions for this protein have been disclosed [15, 54–56]. Tliba et al. [57] described a new possible mechanism in which cytokine-stimulated IRF-1 leads to competitive recruitment of GRIP-1 in detriment of the GR transcriptional regulatory complexes at the GRE element of promoters, thus leading to attenuated trans-activation by steroids [58]. Whether this mechanism is important in epithelial cells remains to be determined. More recently, an alternative role or function for GR- β was discovered [15, 55, 56]; these authors found that GR- β possess inherent transcriptional regulatory activity observed by the up-regulation or the

down-regulation of many target genes distinct to those regulated by GR- α . This finding suggests that important physiological/biochemical changes could be triggered by GR- β despite its lower abundance in some cells and tissues compared with GR- α . Although the molecular mechanisms remain unclear, whether GR- β 's regulation of target genes alter the cell's steroid responsiveness requires further investigation.

Acknowledgements

This work was supported by the Richard and Edith Strauss Foundation (Montreal, QC, Canada), the Canadian Institutes of Health Research (Ottawa, ON, Canada) and the J. T. Costello Memorial Research Fund (Montreal, QC, Canada) to Q. H., and by an NIAID 1R01AI070140 fund to D. Y. L.

Disclosure: All the authors have declared that they have no conflict of interest.

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