

ORIGINAL ARTICLE

The role of interleukin-4R α in the induction of glutamic acid decarboxylase in airway epithelium following acute house dust mite exposure

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Summary

Background Asthma is a disease characterized by airway inflammation, remodelling and dysfunction. Airway inflammation contributes to remodelling, a term that is used to describe structural changes including goblet cell metaplasia (GCM), matrix deposition, and smooth muscle hyperplasia/hypertrophy. GCM has been implicated in asthma mortality by contributing to mucus plugs and leading to asphyxiation. In animal models, this process is highly dependent on IL-13. Recently, we have described an IL-13-dependent up-regulation of a GABAergic signalling system in airway epithelium that contributes to GCM. The mechanism by which IL-13 up-regulates GABA signalling in airway epithelium is unknown.

Objectives To test the hypothesis that IL-4R α signalling is required for allergen induced up-regulation of GABAergic signalling and GCM.

Methods BALB/c mice were exposed to an acute house dust mite (HDM) protocol and received vehicle, anti-IL-4R α -monoclonal antibody, or control antibody. Outcomes included airway responses to inhaled methacholine (MCh), histology for eosinophilia and GCM, phosphorylated STAT6 levels using immunohistochemistry and immunoblot, and glutamic acid decarboxylase (GAD) 65/67 and GABA_A $\beta_{2/3}$ receptor subunit expression using confocal microscopy.

Results Acute HDM exposure resulted in increased airway responses to MCh, lung eosinophilia, STAT6 phosphorylation, elevations in GAD65/67 and GABA_A $\beta_{2/3}$ receptor expression, and GCM that were inhibited with anti-IL-4R α -monoclonal treatment. Control antibody had no effect.

Conclusion The IL-4R α is required for allergen-induced up-regulation of a GABAergic system in airway epithelium implicated in GCM following acute HDM exposure.

Keywords airway remodelling, asthma, GABA, goblet cells, mouse

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Introduction

Asthma is a disease characterized by Th2 skewed airway inflammation, remodelling and hyperresponsiveness. The efficacy of anti-inflammatory treatments in controlling airway function of humans has provided evidence to support the contributing role that inflammation plays in the development of airway dysfunction [1, 2]. Inflammation is accompanied by remodelling of the airway wall and the two have been positively associated in animal studies [3, 4], although causal links attained from clinical data remain elusive.

Airway remodelling is a broad term that encompasses several changes in the airway wall composition including

goblet cell metaplasia (GCM), increased matrix deposition, smooth muscle hyperplasia, and epithelial desquamation [4]. The relative contribution of each airway remodelling change to airway hyperresponsiveness (AHR) is not clear, although it has been demonstrated that GCM and mucus overproduction contribute to fatal asthma attacks by forming mucus plugs [4]. Experimental mouse models of asthma using transgenics, KOs, and acute and chronic allergen exposure protocols have implicated the Th2 cytokine IL-13 in the development of GCM [3, 5–7].

IL-4 and IL-13 cytokines share the IL-4R α chain to form their functionally signalling receptors [8–10]. IL-4 is capable of signalling through two distinct receptors that utilize the IL-4R α chain. The type I IL-4 receptor consists

of a common γ -chain and the IL-4R α , while the type II IL-4 receptor consists of the IL-4R α and the IL-13R α 1. Docking of STAT6 to phosphorylated tyrosine residues on the IL-4R α results in STAT6 phosphorylation by janus-activated tyrosine kinases and subsequent signalling through the nuclear import of phosphorylated STAT6 homodimers [8, 10, 11]. Many genes associated with allergy contain STAT6-binding motifs in their upstream promoter regions including eotaxin, IL-4, IL-4R α , and IL-13R α 2. Recently, our group has characterized distinct patterns of STAT6 phosphorylation in two different strains of mice that are associated with disparate outcomes in GCM [12].

The clear role for IL-13 in the development of GCM has been defined in several key studies [3, 6, 7]. Subsequent studies inhibiting or knocking out the IL-4R α chain or STAT6 support the notion that IL-13 is mediating allergic responses primarily through the type II IL-4 receptor/STAT6 in a classical manner [13–18]. Recently, we have described an IL-13 dependent up-regulation of a GABAergic signalling system in airway epithelium, which is required for GCM [19]. The production of GABA is regulated by two isoforms of glutamic acid decarboxylase (GAD), known as GAD65 and GAD67 [20]. The GABA produced in turn can bind to ionotropic GABA_A or metabotropic GABA_B receptors. In an acute ovalbumin exposure model, we have demonstrated that the GAD65/67 protein expression is elevated and mediates responses through the GABA_A receptor [19]. Classically, GABA_A receptor activity in the CNS is inhibitory, yet we have demonstrated that in the airway epithelium GABA activation of this receptor is excitatory. It is unknown, however, if this mechanism of GABAergic-mediated GCM is mediated through the Type II IL-4 receptor and requires classical STAT6 activation [21]. We set out to test the hypothesis that allergen-mediated up-regulation of a GABAergic signalling system in airway epithelium is mediated through the IL-4R α chain. By using a monoclonal antibody to IL-4R α (anti-IL-4R α mAb) in a model of acute house dust mite (HDM) exposure and examining the airway physiology, inflammation, STAT6 phosphorylation, expression of genes containing STAT6-binding motifs (IL-4, IL-13R α 2), GAD65/67 protein expression, and GCM.

Materials and methods

Animals

Female BALB/c wild-type mice, aged 8–10 weeks, were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). All mice were housed in environmentally controlled, specific pathogen-free conditions for a 1-week acclimatization period and throughout the duration of the studies. All procedures were approved by the Animal Research Ethics Board at the McMaster University, and conformed to the NIH guidelines for the experimental use of animals.

Acute house dust mite allergen exposure model and intervention design

Four groups of animals ($n = 10$) were studied as follows: (1) Saline exposed/vehicle intervention, (2) HDM exposed/vehicle intervention, (3) HDM exposed/anti-IL-4R α mAb intervention, and (4) HDM exposed/control antibody intervention. HDM extract (Greer Laboratories, Lenoir, NC, USA) was prepared as described previously [22]. Mice received 15 μ g of HDM extract in 25 μ L phosphate-buffered saline (PBS) volume on Days 1–5 and 8–12 (Fig. 1) under gaseous anaesthesia via the intranasal (IN) route. Control animals received IN saline exposure. Groups 1 and 2 received 250 μ L of PBS via an intraperitoneal (IP) injection as vehicle treatment. Group 3 received 250 μ L of a 4 mg/mL anti-IL-4R α mAb (Amgen, Seattle, WA, USA) via an IP injection [23]. Group 4 received 250 μ L of a 4 mg/mL control mAb (Amgen) via an IP injection. IP injections were performed on Days 1, 5, and 10 based on pharmacokinetic data (data not shown). Outcome measurements were made 72 h post final HDM exposure (Day 15).

Airway responsiveness

Airway responsiveness was measured using the flexiVent small animal ventilator system (SciReq, Montreal, Quebec, Canada). The single compartment model was used and the total respiratory system resistance (R_{RS}) in response to nebulized saline and increasing doses of methacholine (MCh) ($n = 10$ per group) was assessed.

Bronchoalveolar lavage and serum analysis

BAL cell differential analysis was performed as described previously [12].

Lung histology

Lungs were dissected and the left lobes were inflated, fixed in formalin, and embedded in paraffin as described previously [12]. Three-micron-thick sections were stained with Mayer's haematoxylin/eosin (H&E) and periodic acid–Schiff (PAS) for assessing the presence of tissue eosinophils and goblet cells, respectively. Sections were immunostained using antibodies against phospho-STAT6 (pSTAT6) (Abcam, Cambridge, MA, USA) and GAD65/67 (Sigma, Mississauga, Ontario, Canada). All immunostains required antigen retrieval in citrate buffer (pH 6.0) in a steamer at 90 °C for 20 min followed by cooling of slides to room temperature for 20 min. Blocking was performed with 1% normal swine serum for 30 min at room temperature and all subsequent reagents were dissolved in this solution. Primary antibodies were diluted at 1:100, 1:800, 1:100 for pSTAT6, GAD 65/67, and GABA $\beta_{2/3}$.

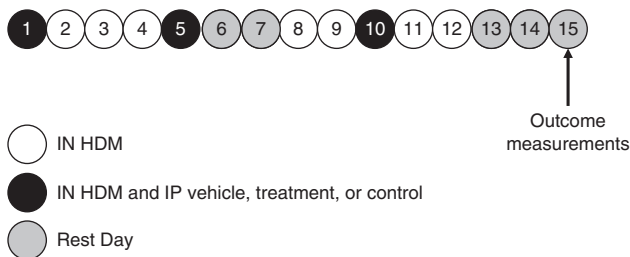


Fig. 1. Allergen sensitization and intervention dosing protocol. Four groups of animals ($n = 10$) were studied (see Materials and methods). Saline exposed/vehicle intervention, house dust mite (HDM) exposed/vehicle intervention, HDM exposed/anti-IL-4R α mAb intervention, and HDM exposed/control antibody intervention. Black circles, intranasal (IN) HDM and intraperitoneal injection of vehicle, anti-IL4Ra-mAb intervention, or control antibody intervention; open circles, IN HDM exposure; grey circles, rest day.

respectively, and incubated overnight at 4 °C. For pSTAT6 staining, biotinylated secondary antibodies were added at 1 : 100 concentrations, while streptavidin peroxidase was added at 1 : 600 concentration. Colour development was performed by placing slides in a 0.4% aminoethylcarbazole/dimethylformamide solution for 30 min, initiated by the addition of four drops of H₂O₂. Counterstaining was performed with Mayer's haematoxylin. For GAD65/67 and GABA $\beta_{2/3}$ staining, an FITC-conjugated antibody to pan-cytokeratin (1 : 100 dilution; Sigma) was used to visualize epithelium. A Cy3-conjugated secondary antibody was used to detect GAD65/67 and GABA $\beta_{2/3}$ staining (1 : 100 dilution; Sigma).

Lung morphometry

PAS-stained tissue sections were viewed and images were collected under $\times 20$ objective magnification light microscopy as described previously [12]. H&E-stained tissue sections were viewed and images were collected under a $\times 40$ objective. Eosinophils were expressed as number/area in a 50 μ m band extending from the basement membrane of the large primary bronchus as described previously [12]. Confocal images of GAD65/67 and GABA $\beta_{2/3}$ staining of airway epithelium were analysed with morphometry as follows: the area of GAD65/67 or GABA $\beta_{2/3}$ staining stain (red) was determined and expressed as a ratio of the airway epithelium area. Multiple images were collected for each animal with an n value equal to or greater than 4.

Immunoblots

Immunoblots were performed as described previously [12]. An immunoblot primary antibody specific for phosphotyrosine pY641 STAT6 (pSTAT6) was purchased from Cell Signalling Technology Inc. (Danvers, MA, USA). Primary antibodies (Abs) specific for actin (I-19) were

purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary Abs against pSTAT6 were diluted 1/1000 in 5.0% bovine serum albumin (BSA; Sigma-Aldrich, Oakville, Ontario, Canada). For the detection of primary pSTAT6 and STAT6, secondary goat anti-mouse IgG horse radish peroxidase (HRP) Ab was purchased from Santa Cruz Biotechnology. Goat anti-mouse IgG HRP Ab was diluted 1/2500 in 5.0% milk. For the detection of primary actin (I-19) Ab, rabbit anti-goat IgG HRP Ab was purchased from Sigma-Aldrich. Rabbit anti-goat IgG HRP Ab was diluted 1/2500 in 5.0% milk.

Whole mouse lung tissue was homogenized in a radio-immunoprecipitation (RIPA) lysis buffer [$1 \times$ PBS (pH 7.4), 1.0% igepal CA-630, 0.5% sodium deoxycholate (C₂₄H₃₉NaO₄), 0.1% SDS, including inhibitors (1 mM PMSF, 1 mM sodium orthovanadate (Na₃VO₄), and 1 mg/mL aprotinin]. Lung homogenates were incubated for 1 h on ice. Lung homogenates were then vigorously sheared using a syringe with a 21-gauge needle, centrifuged at maximum speed (12,000 g) at 4 °C, supernatant collected, and stored at -70 °C. Protein concentration was determined using the Bio-Rad Protein assay (Bio-Rad, Mississauga, Ontario, Canada). Equal amounts of protein were loaded to 8% SDS-PAGE gels for electrophoresis. Proteins were transferred to Immobilon-NC membranes (Millipore, Ottawa, Ontario, Canada). Immunoblots were blocked in $1 \times$ TBS containing 0.15% Tween 20 and 5.0% fat-free milk powder for 1 h at room temperature and probed with primary Abs overnight at 4 °C. Membranes were washed, incubated with HRP-conjugated secondary Abs for 1 h at room temperature, and visualized using an ECL Western Blotting Analysis System (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada). Immunoblots were exposed by X-OMAT Kodak Scientific Films and images scanned at 300–400 dpi resolution.

Gene expression analysis

Following euthanasia, lungs were removed, rinsed in PBS, and immediately immersed in RNAlater (Ambion, Streetsville, ON, Canada) and stored at 4 °C overnight as per the manufacturer's recommendations. RNA isolation was performed using a Qiagen MiniPrepKit (Valencia, CA, USA) and the concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was transcribed using a standard protocol with an ABI High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was conducted using custom Taqman Low Density Arrays (Applied Biosystems) read on an ABI Prism 7900HT Sequence Detector (Applied Biosystems). Relative quantification was determined using the comparative C_t method. Expression levels were calculated relative to the expression of the control gene hypoxanthine guanine phosphoribosyltransferase (HPRT).

GAD67 gene expression analysis was performed using the primer set Mm000725661 and normalized to GAPDH (Applied Biosystems).

Statistical analysis

Summary data used in all comparisons are expressed as mean and standard error of the mean (SEM). Comparisons between saline control and OVA-exposed mice for each were made using a one-way ANOVA with a Newman-Keuls *post hoc* test. Differences were assumed to be statistically different when the observed *P* values were <0.05 .

Results

Anti-interleukin-4R α monoclonal antibody treatment inhibits acute house dust mite-induced airway hyperresponsiveness and lung eosinophilia

Acute HDM exposure resulted in AHR ($P<0.05$; supporting information Fig. S1a) illustrated by increased maximum respiratory resistance ($P<0.05$; supporting information Fig. S1b) measured 72 h post final allergen exposure. Intervention with anti-IL-4R α mAb significantly attenuated the HDM-induced AHR ($P<0.05$; supporting information Figs S1a and b). Control mAb intervention had no effect on AHR.

Acute HDM exposure resulted in increased airway ($P<0.05$; Fig. 2a) and tissue ($P<0.05$; Fig. 2b) eosinophils. Intervention with anti-IL-4R α mAb attenuated HDM-induced airway and tissue eosinophilia ($P<0.05$; Figs 2a and b), while control mAb had no effect. Gene expression analysis of IL-5 and eosinophil peroxidase were also significantly elevated following acute HDM exposure ($P<0.05$; Figs 2c and d), which was attenuated with anti-IL-4R α mAb treatment. Attenuated airway and tissue eosinophils, and IL-5 and eosinophil peroxidase gene expression, were significantly elevated above control animal levels in anti-IL-4R α mAb-treated mice (Fig. 2), suggesting that our intervention strategy did not prevent allergic sensitization but instead diminished allergic responses.

Anti-interleukin-4R α monoclonal antibody treatment inhibits acute house dust mite-induced lung STAT6 phosphorylation

We assessed IL-4R α signalling by examining phosphorylated STAT6 (pSTAT6) levels using immunohistochemistry and immunoblot methods as described previously for ovalbumin exposure models [12]. Representative images of pSTAT6 staining ($n = 4$ per group) are illustrated in Figs 3a–d. Acute HDM exposure resulted in pSTAT6 positively

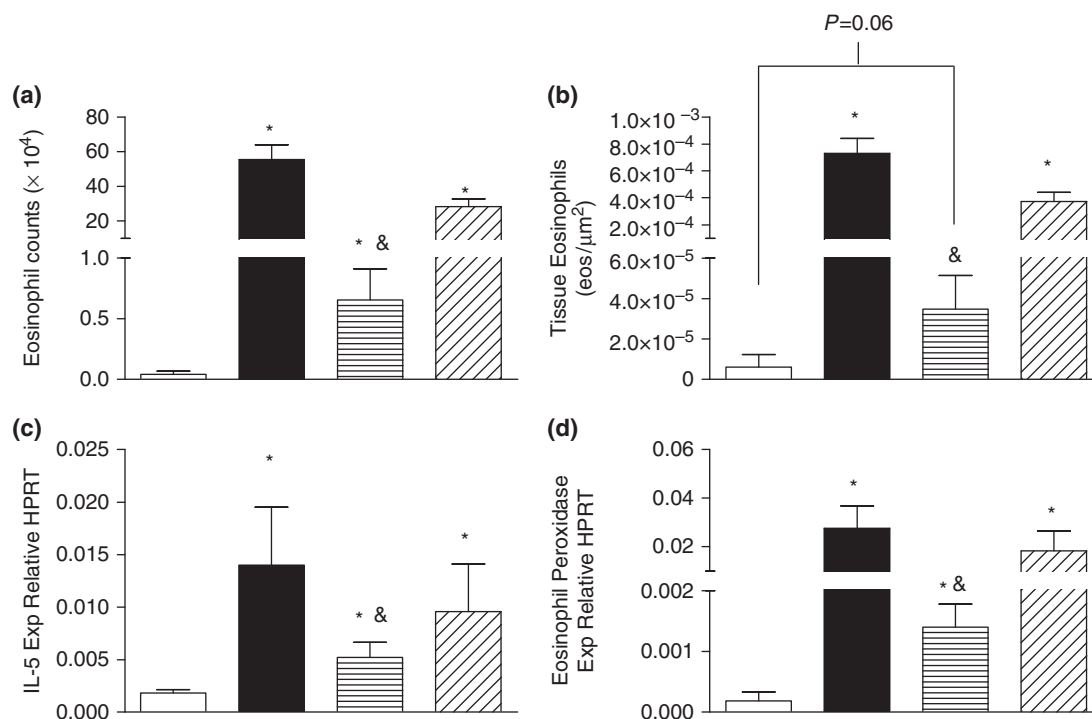


Fig. 2. Airway eosinophilia and expression of genes related to allergic airway inflammation. (a) BAL eosinophils. (b) Tissue eosinophils. (c) Relative gene expression for IL-5. (d) Relative gene expression for eosinophil peroxidase. Open white bar, saline exposed/vehicle intervention; solid black bar, house dust mite (HDM) exposed/vehicle intervention; horizontal dashed white bar, HDM exposed/anti-IL-4R α mAb intervention; diagonal dashed white bar, HDM exposed/control antibody intervention. *Significantly different from saline exposed/vehicle intervention ($P<0.05$). &Significantly different from HDM exposed/vehicle intervention ($P<0.05$).

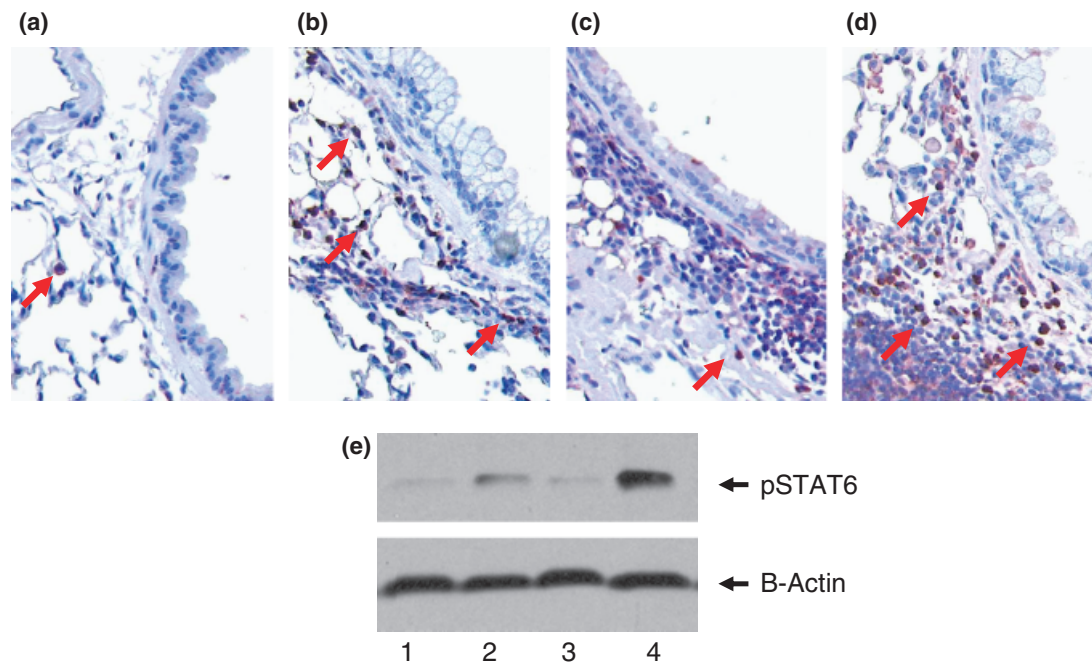


Fig. 3. Expression of the phosphorylated STAT6 (pSTAT6) protein following acute house dust mite (HDM) exposure. Panels (a)–(d): representative images of pSTAT6 immunohistochemistry of mouse lung. Immunostain was developed with aminoethylcarbazole producing a red/brown stain on pSTAT6-positive cells. Slides were counterstained with Mayer's haematoxylin. (a) Saline exposed/vehicle intervention. (b) HDM exposed/vehicle intervention. (c) HDM exposed/anti-IL-4R α mAb intervention. (d) HDM exposed/control antibody intervention. Red arrows point to positively stained cells. (e) Immunoblot of homogenized lung tissue for pSTAT6 following acute HDM exposure. Lane (1) Saline exposed/vehicle intervention. Lane (2) HDM exposed/vehicle intervention. Lane (3) HDM exposed/anti-IL-4R α mAb intervention. Lane (4) HDM exposed/control antibody intervention.

staining in small nuclear dense inflammatory cells within the airway parenchyma and beneath the basement membrane (Figs 3a and b). Anti-IL-4R α mAb intervention inhibited acute HDM-induced elevations in pSTAT6 positively stained cells, while control mAb had no effect (Figs 3c and d). Confirmation of pSTAT6 immunohistochemistry was performed by assessing immunoblotting for pSTAT6 protein levels in whole lung homogenate (Fig. 3e). Acute HDM exposure resulted in elevations in pSTAT6 protein levels (Lane 2) as compared with the saline control (Lane 1). Consistent with immunohistochemistry results, anti-IL-4R α mAb treatment inhibited HDM-induced elevations in pSTAT6 (Lane 3). Control antibody treatment demonstrated no inhibition of pSTAT6 staining (Lane 4).

Anti-interleukin-4R α monoclonal antibody treatment inhibits house dust mite-induced up-regulation of genes containing STAT6-binding motifs – interleukin-4 and -13R α 2

STAT6-binding motifs are contained in the promoters of various genes including IL-4 and IL-13R α 2 [8]. Acute HDM resulted in elevated transcript levels encoding IL-4 and IL-13R α 2 that were attenuated with anti-IL-4R α mAb intervention ($P < 0.05$; Figs 4a and b). Control antibody

had no effect on HDM-induced IL-13R α 2 expression (Fig. 4a), but demonstrated a trend ($P = 0.09$) for a reduction in IL-4 gene transcript levels (Fig. 4b).

Acute house dust mite induces epithelial protein expression of glutamic acid decarboxylase 65/67 which is inhibited by anti-IL-4R α mAb treatment

We have described previously the up-regulation of GAD 65/67 expression in airway epithelium that is involved in an IL-13-dependent GCM in an ovalbumin model of allergen exposure [19]. Confocal microscopy of GAD 65/67 expression in airway epithelium following acute HDM exposure demonstrates an up-regulation following acute HDM allergen exposure (Figs 5a and b). Acute HDM-induced up-regulation of GAD65/67 is inhibited by anti-IL-4R α mAb (Fig. 5c) but not the control antibody (Fig. 5d). Morphometric analysis of airway epithelium demonstrated a HDM-induced up-regulation in area of GAD65/67 staining that was significantly reduced by anti-IL-4R α mAb treatment ($P < 0.05$, Fig. 5e). Gene expression analysis was performed for GAD67 to complement the IHC data. Acute HDM exposure resulted in a significant decrease in GAD67 mRNA levels ($P < 0.05$, Fig. 5f). Anti-IL-4R α mAb treatment inhibited HDM-induced

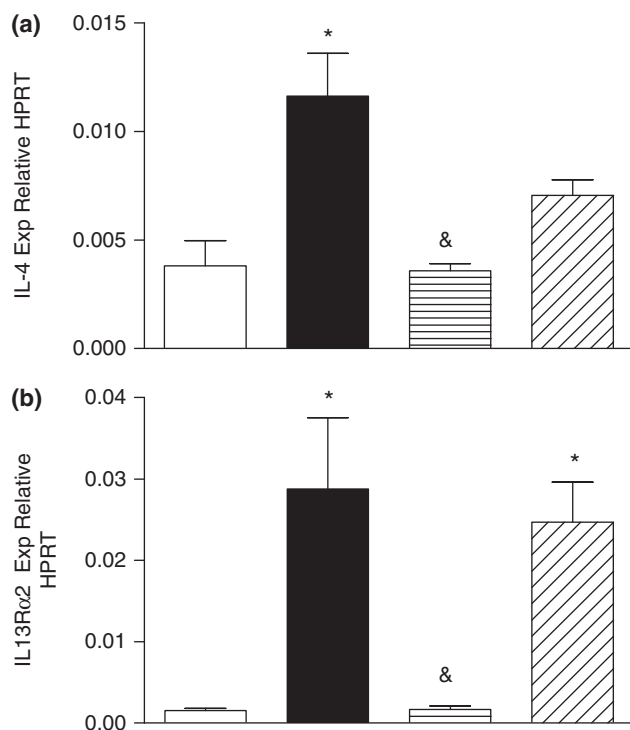


Fig. 4. Relative gene expression of genes containing STAT6-binding motifs following acute house dust mite (HDM) exposure. Gene expression is expressed relative to hypoxanthine guanine phosphoribosyltransferase (HPRT, see Materials and methods). (a) IL-4 (b) IL-13R α 2. Open white bar, saline exposed/vehicle intervention; solid black bar, HDM exposed/vehicle intervention; horizontal dashed white bar, HDM exposed/anti-IL-4R α mAb intervention; diagonal dashed white bar, HDM exposed/control antibody intervention. *Significantly different from saline exposed/vehicle intervention ($P < 0.05$). &Significantly different from HDM exposed/vehicle intervention ($P < 0.05$).

changes in GAD67 expression, while the control antibody had no effect (Fig. 5f).

Acute house dust mite induces epithelial expression of GABA $\beta_{2/3}$ receptor subunit expression

Confocal microscopy of GABA $\beta_{2/3}$ receptor subunit expression in airway epithelium demonstrates an up-regulation following acute HDM allergen exposure ($P < 0.05$, Figs 6a and b). Morphometric analysis of airway epithelium demonstrated a HDM-induced up-regulation in the area of the GABA receptor $\beta_{2/3}$ subunit staining. Anti-IL-4R α mAb treatment demonstrated a trend for reduced GABA receptor $\beta_{2/3}$ subunit staining ($P = 0.09$, Fig. 6e).

Anti-IL-4R α mAb treatment inhibits acute house dust mite-induced goblet cell metaplasia and mucin gene expression

Acute HDM exposure resulted in elevations in PAS positively stained cells with goblet cell morphology as com-

pared with saline exposure (Figs 7a and b). Anti-IL-4R α mAb treatment inhibited the development of PAS positively stained goblet cells, although some PAS-positive cells without goblet cell morphology were observed (Fig. 7c). Control antibody had no effect (Fig. 7d).

Quantification of positively stained PAS cells revealed a HDM-induced increase, which was significantly attenuated by anti-IL-4R α mAb treatment ($P < 0.05$; Fig. 7e). Control antibody had no treatment effect.

Consistent with GCM, acute HDM induced increases in MUC5AC ($P < 0.05$; Fig. 7f) and Gob5 gene expression ($P < 0.05$; Fig. 7g). Anti-IL-4R α mAb treatment significantly attenuated acute HDM-induced changes in mucin gene expression ($P < 0.05$; Figs 7f and g).

Discussion

The present results extend our previous findings of an IL-13-dependent GABAergic signalling system in airway epithelium involved in GCM in an ovalbumin model of allergen exposure [19]. We demonstrate by using acute HDM exposure that the up-regulation of a GABAergic system in airway epithelium is conserved in different models of allergen exposure. Furthermore, we demonstrate that acute HDM results in phosphorylation of STAT6, a key signalling molecule downstream of IL-4R α . Finally, we provide mechanistic evidence using an anti-IL-4R α mAb intervention that HDM induced response signals through IL-4R α to produce elevations in STAT6 phosphorylation, GAD65/67 expression, GCM, and mucus gene expression.

The signalling cascade of IL-13 leading to an up-regulation of the GABAergic system in airway epithelium and GCM is not fully elucidated [21]. IL-13 is capable of signalling through a heterodimer of IL-4R α and IL-13R α 1 [24] or through IL-13R α 2 [25]. Previous reports using IL4R $\alpha^{-/-}$ mice demonstrate that this receptor is necessary for GCM following ovalbumin exposure [6, 18]. Our data using a pharmacological inhibitor of the IL-4R α are consistent with these findings and extends them by demonstrating that GAD 65/67 expression in models of allergen exposure is regulated through the IL-4R α and downstream STAT6 pathway.

STAT6 is a transcription factor downstream of the receptor chain IL-4R α and can be phosphorylated as a result of IL-4 or IL-13 signalling [8]. IL-4 may signal through STAT6 using the IL-4R α and the common γ receptor chain, and IL-13 uses the IL-13R α 1 chain in place of the common γ chain. Using ovalbumin exposure models, we have demonstrated previously that STAT6 phosphorylation observed is dependent on IL-13 [12]. Our findings of elevated pSTAT6 levels following acute HDM exposure are consistent with those observed in ovalbumin models. It remains to be determined if changes in pSTAT6 levels in HDM models are IL-13 dependent,

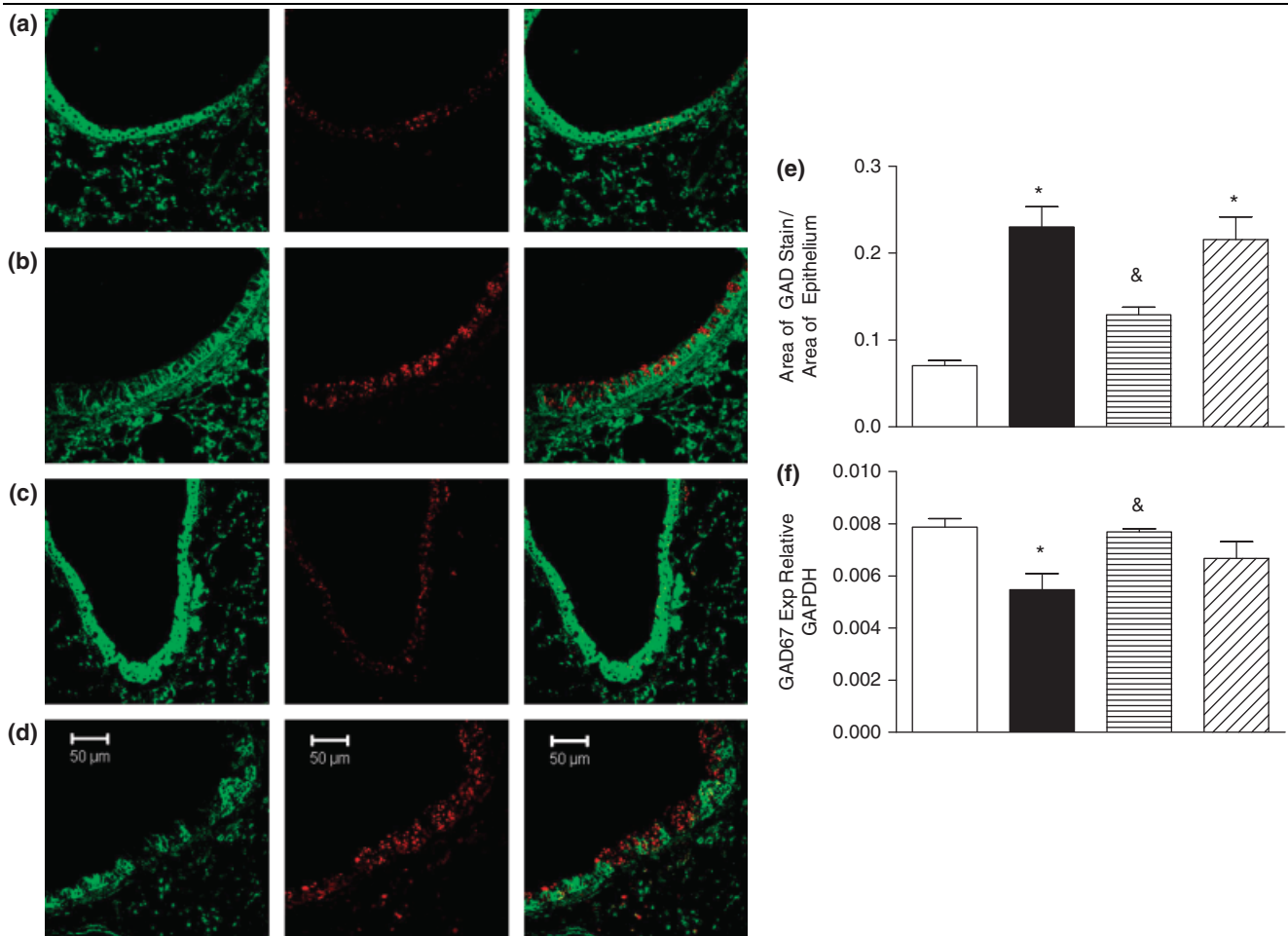


Fig. 5. Expression of GAD65/67 protein in mouse lung using confocal microscopy following acute house dust mite (HDM) exposure. Epithelial cells were identified by immunostaining with pan-cytokeratin (green). GAD 65/67 immunostaining is red. Representative images ($n = 17$ per group). (a) Saline exposed/vehicle intervention. (b) HDM exposed/vehicle intervention. (c) HDM exposed/anti-IL-4R α mAb intervention. (d) HDM exposed/control antibody intervention. (e) Morphometric analysis of GAD65/67 staining. Fifteen animals from each group were analysed. The area of GAD65/67 staining was normalized to the epithelium area. (f) Relative gene expression of GAD67 following acute HDM exposure. Gene expression is expressed relative to GAPDH. Open white bar, saline exposed/vehicle intervention; solid black bar, HDM exposed/vehicle intervention; horizontal dashed white bar, HDM exposed/anti-IL-4R α mAb intervention; diagonal dashed white bar, HDM exposed/control antibody intervention. *Significantly different from saline exposed/vehicle intervention ($P < 0.05$). &Significantly different from HDM exposed/vehicle intervention ($P < 0.05$). GAD, glutamic acid decarboxylase.

although the staining pattern, cell morphology, and expression levels are similar to IL-13 dependent STAT6 elevations observed in OVA models [12].

The functional consequences of IL-4R α blockade have been reported using models of ovalbumin exposure with knockout of the IL-4R α [6, 18], while indirectly explored in STAT6-deficient animals [15, 26]. Classical a knockout animal studies suffer from the potential for compensatory mechanisms and potentially confounding animal development issues [27] although the use of cre-lox inducible knockouts circumvent these problems [28]. Using our acute model of HDM exposure, we support the role of IL-4R α in the development of allergen induced AHR, although the precise mechanisms by which this occurs are unknown. We provide a proof of concept study for IL-

4R α intervention in chronic models of HDM exposure, which will allow a clinically relevant assessment of this pathway, including the treatment initiating following the establishment of airway remodelling and sustained AHR [29]. Chronic models of IL-4R α intervention using antisense technology have observed attenuation in the allergic phenotype and AHR – but a residual GCM still exists [17].

Similar to Karras et al. [17], we did not see complete attenuation of GCM in our anti-IL-4R α mAb-treated mice. It is possible that our pharmacological inhibitor did not achieve complete receptor blockade of acute HDM-induced signalling through the IL-4R α pathway, although published reports using this reagent at similar doses had efficacy in an allergic gastrointestinal model [23]. We

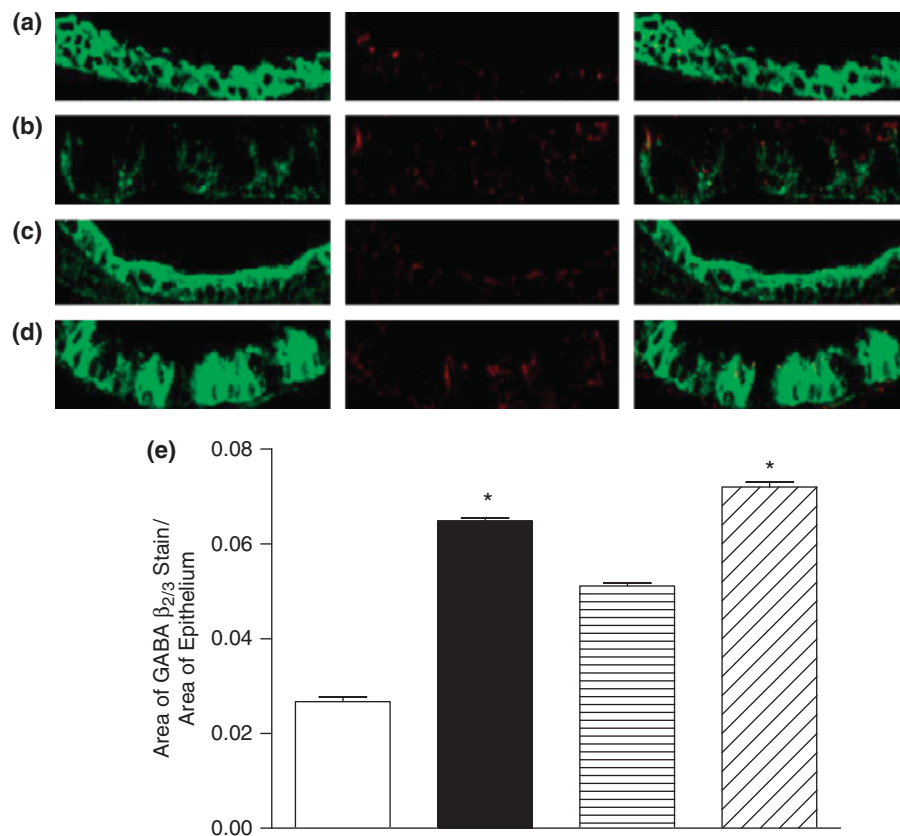


Fig. 6. Expression of the GABA_A β_{2/3} receptor subunit expression in mouse lung using confocal microscopy following acute house dust mite (HDM) exposure. Epithelial cells were identified by immunostaining with pan-cytokeratin (green). GABA_A β_{2/3} immunostaining is red. (a) Control saline exposed/vehicle intervention. (b) HDM exposed/vehicle intervention. (c) HDM exposed/anti-IL-4Rα mAb intervention. (d) HDM exposed/control antibody intervention. (e) Morphometric analysis of GABA_A β_{2/3} receptor subunit staining. Multiple images of airways ($n = 3-6$) from at least four animals from each group were analysed. The area of the GABA_A β_{2/3} receptor subunit staining was normalized to epithelium area. *Significantly different from saline exposed/vehicle intervention ($P < 0.05$).

examined this possibility by assessing IL-4 and IL-13Rα2 gene transcript levels, two genes reported to be regulated by the IL-4Rα/STAT6 pathway [8]. Complete inhibition of acute HDM-induced elevations in IL-4 and IL-13Rα2 gene transcript suggest that we had sufficient levels of inhibitor to block gene expression and this was further supported by the normalized pSTAT6 protein expression.

We have reported previously on a GABAergic signalling system in airway epithelium involved in GCM that is dependent on IL-13 [19]. Ovalbumin exposure models were used in animal studies to support these findings. As ovalbumin exposure models require artificial sensitization [30], they may lead to allergen tolerance [31], and are not reflective of clinically relevant allergens [32–34], we sought to examine whether up-regulation of the GABAergic signalling system was conserved for more relevant allergens. Using a model of acute HDM exposure that requires no artificial sensitization, results in no allergen tolerance [22, 33, 35], and is of clinical relevance [36], we

demonstrate that GABAergic up-regulation in airway epithelium is conserved among two common models of allergen exposure.

Our HDM data are consistent with our previous publication that demonstrates an up-regulation in GABA receptor β_{2/3} subunits in response to acute ovalbumin exposure [19]. The trend for decreased HDM-induced GABAβ_{2/3} receptor subunit expression in response to anti-IL-4Rα mAb treatment is consistent with a reduction in the GAD65/67 expression, although the former did not reach significance. Ultimately, the activity of the GABAergic system depends on the balance between the GAD65/67 generation of GABA and GABAβ_{2/3} receptor subunit expression. It is conceivable that the anti-IL-4Rα mAb treatment mediated a reduction in GAD65/67, but not the GABAβ_{2/3} subunit expression, is sufficient to reduce GABAergic-mediated GCM.

GABA has both ionotropic GABA_A and metabotropic GABA_B receptors. We first reported the expression of the

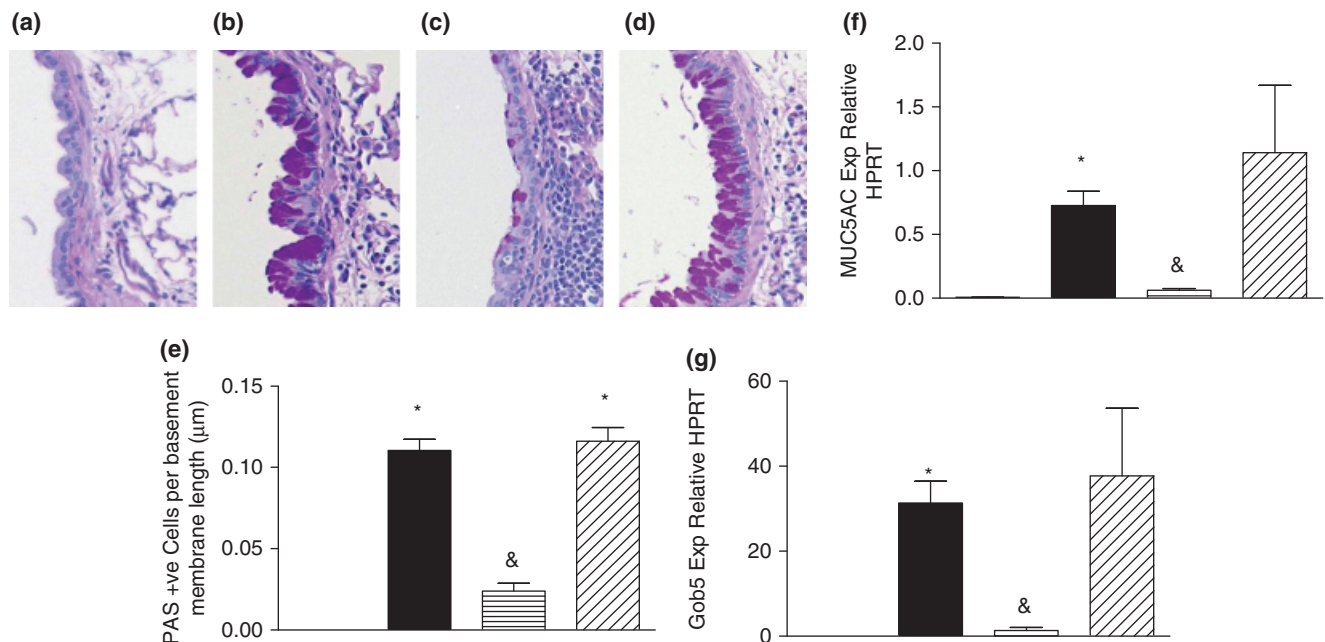


Fig. 7. Goblet cell analysis and the relative gene expression of mucus-related genes following acute house dust mite (HDM) exposure. Representative photomicrographs of periodic acid-Schiff (PAS) positively stained slides for (a) saline exposed/vehicle intervention, (b) HDM exposed/vehicle intervention, (c) HDM exposed/anti-IL-4R α mAb intervention, and (d) HDM exposed/control antibody intervention. (e) Quantification of PAS positively stained cells. Gene expression relative to hypoxanthine guanine phosphoribosyltransferase (HPRT, see Materials and methods). (f) MUC5AC. (g) Gob5. Open white bar, saline exposed/vehicle intervention; solid black bar, HDM exposed/vehicle intervention; horizontal dashed white bar, HDM exposed/anti-IL-4R α mAb intervention; diagonal dashed white bar, HDM exposed/control antibody intervention. *Significantly different from saline exposed/vehicle intervention ($P < 0.05$). &Significantly different from HDM exposed/vehicle intervention ($P < 0.05$).

GABA $_A$ receptors in the airway epithelium of mice and humans and the relationship with GCM in asthma [19]. We were unable to localize the GABA $_A$ receptor expression to any other tissue, confirmed in the present study. Recently, Mizuta et al. [37] have identified the functional expression of GABA $_B$ receptors in human and guinea-pig airway epithelium. Collectively, these results suggest that a diverse GABAergic system is operating in both normal and disease states in the airway epithelium.

Our attempt to complement our protein expression data of GAD65/67 with gene expression analysis has produced some paradoxical results. While we demonstrate increases in protein expression with acute HDM exposure, an opposite pattern is observed at the gene expression level for GAD67. It is possible that increases in GAD67 protein and the related production of GABA generate a negative feedback loop that reduces the expression of GAD67. Alternatively, it is possible that the major protein expression we observe in airway epithelium is the result of GAD65, a gene that we were unable to detect with two distinct primer sets.

Our findings provide mechanistic evidence to support the role of an IL-4R α -dependent GABAergic signalling system in airway epithelium involved in GCM in response to allergen exposure. Using an anti-IL-4R α mAb interven-

tion, we demonstrate for the first time that allergic responses signal through this receptor chain to up-regulate GAD 65/67 expression in airway epithelium and induce GCM. Consistent with the inhibition of IL-4R α signalling, we observe reduced phosphorylated STAT6 protein levels with anti-IL-4R α mAb treatment during HDM exposure. These findings support the pursuit of IL-4R α inhibitors for the management of allergen-induced GCM and the associated airway dysfunction.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Airway responsiveness to nebulized methacholine. a) Dose response curve. Black circles/solid line: Saline exposed/vehicle intervention (see methods). Black squares/solid line: HDM exposed/vehicle intervention. Open circles/dashed line: HDM exposed/anti-IL-4R α mAb intervention. Open squares/dashed line: HDM exposed/control antibody intervention. b) Maximum airway resistance. Open white bar: Saline exposed/vehicle intervention. Solid black bar:

HDM exposed/vehicle intervention. Horizontal dashed white bar: HDM exposed/anti-IL-4R α mAb intervention. Diagonal dashed white bar: HDM exposed/control antibody intervention. * = significantly different from saline exposed/vehicle intervention ($P < 0.05$). $\S$ = significantly different from HDM exposed/vehicle intervention ($P < 0.05$).

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