

Dissociation of T helper type 2 cytokine-dependent airway lesions from signal transducer and activator of transcription 6 signalling in experimental chronic asthma

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Summary

Background Type 2 T helper lymphocytes (Th2 cells) and their cytokine products are important in the pathogenesis of asthma.

Objective To examine the contribution of the signal transducer and activator of transcription (STAT) 6 pathway, involved in Th2 cytokine signalling, to the development of lesions of chronic asthma.

Methods BALB/c mice sensitized to ovalbumin were chronically challenged by inhalational of low mass concentrations of antigen for 6 weeks. Airway lesions in wild-type mice were compared with those in STAT6-deficient mice and in IL-4/13 double-deficient mice by histomorphometry and immunohistochemistry. Airway responses to methacholine were evaluated by whole-body plethysmography. Cytokine production by peribronchial lymph node cells was quantified by enzyme immunoassay.

Results STAT6^{−/−} mice developed a variety of airway lesions that were at least equivalent to those in wild-type mice, including accumulation of intraepithelial eosinophils and of chronic inflammatory cells in the lamina propria, subepithelial fibrosis and epithelial thickening. In addition, STAT6^{−/−} mice exhibited exaggerated airway hyper-reactivity (AHR) compared to wild-type animals. This was despite a shift from a Th2 to a Th1 pattern of immunoglobulin production by plasma cells in the inflammatory infiltrate and diminished mucous cell hyperplasia/metaplasia, together with increased production of IFN- γ by peribronchial lymph node cells, consistent with absence of signalling via the STAT6 pathway. In contrast, gene-targeted IL-4/13^{−/−} mice exhibited markedly diminished eosinophil recruitment and airway remodelling, as well as absence of AHR.

Conclusions In this model, the effects of STAT6 deficiency were in marked contrast to the suppression of inflammation and AHR described in models of allergic bronchopulmonary inflammation. These results, which provide evidence of STAT6-independent AHR in an inhalational challenge model of chronic asthma, emphasize the critical effector roles of IL-4 and IL-13, as well as the need to use appropriate models to understand cytokine signalling pathways that may be potential therapeutic targets in asthma.

Keywords allergy, cytokines, inflammation, *in vivo* animal models, STAT6

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Introduction

Type 2 CD4⁺ T helper lymphocytes (Th2 cells) and their cytokine products, including interleukins 4, 5, 9 and 13, play critically important roles in the pathogenesis of asthma [1]. Investigations in humans suggest that Th2 cytokines are particularly significant in the development of acute-on-chronic airway inflammation and of functional abnormalities including airflow obstruction and airway hyper-reactivity (AHR) [2, 3],

but their contribution to the structural changes of airway wall remodelling is less well understood [4].

Animal models of allergic bronchopulmonary inflammation have been extensively used for experimental studies of Th2 cytokine-mediated disease, but such models have significant flaws with respect to the study of asthma [5]. To overcome these limitations, we have developed an improved model of chronic asthmatic inflammation of the airways in mice [6]. This model replicates most of the features that are typical of the chronic human disease, including accumulation of numerous intraepithelial eosinophils; chronic inflammation in the lamina propria; epithelial hypertrophy, mucous cell hyperplasia/metaplasia and subepithelial fibrosis; and AHR that can be attributed

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to abnormalities of the airways rather than to parenchymal lesions (RA Collins et al. submitted for publication).

We have employed this more realistic model to investigate the pathogenesis of lesions of chronic asthma, using gene targeting and monoclonal antibody depletion to examine the contribution of individual mediators and cellular populations [7–10]. Many of the results we have obtained differ from those in short-term models and several novel findings have emerged, including the demonstration of a hitherto unrecognized role for CD4⁺ T cells in the pathogenesis of airway wall remodelling [9]. These studies have also highlighted two important areas of complexity *viz.* the relative importance of individual Th2 cytokines and their signalling pathways in the development of remodelling and the contribution of these mediators to the pathogenesis of AHR [7, 8]. In the present study, we tested the hypothesis that in chronic asthma, inflammation, remodelling and AHR are abrogated in mice lacking both IL-4 and IL-13, as well as mice in which the signal transducer and activator of transcription (STAT) 6 pathway, involved in signalling for both of these Th2 cytokines [11], is deficient. Our results emphasize the complex, integrated interplay between these mediators and suggest that a simplistic approach of attempting to inhibit individual signalling pathways in asthma is unlikely to yield worthwhile therapeutic outcomes.

Methods

Animals

Wild-type specific pathogen-free female BALB/c mice were obtained from the Biological Resources Centre, University of New South Wales, Sydney, Australia. Specific pathogen-free mice genetically deficient in their ability to produce both IL-4 and IL-13 (IL-4/13^{−/−}) or in their ability to express STAT6 (STAT6^{−/−}) (in each case backcrossed for at least eight generations on to the BALB/c strain) [12] were obtained from the Animal Services Division, Australian National University. Routine serological screening for relevant respiratory pathogens was undertaken by the respective suppliers. Animals were aged approximately 8 weeks at the commencement of experimental studies. They were maintained in a laminar flow holding unit (Gelman Sciences, Sydney, Australia) and housed in autoclaved cages on autoclaved bedding in an airconditioned room on a 12-h light/dark cycle. Irradiated food and acidified water were provided *ad libitum* throughout. All experimental procedures complied with the requirements of the Animal Care and Ethics Committee of the University of New South Wales (ref. no. 01/34.1).

Sensitization and inhalational exposure

This was performed essentially as previously described [6]. Mice were sensitized by an intraperitoneal injection of 50 µg alum-precipitated chicken egg ovalbumin (Grade V, ≥ 98% pure, Sigma, St Louis, MO, USA; unless otherwise specified, all chemicals were obtained from this source) 21 days and 7 days before inhalational exposure. Experimental groups comprising seven to eight sensitized animals were exposed to aerosolized ovalbumin for 30 min/day on 3 days/week for 6 weeks in a whole-body inhalation exposure system (Unifab Corporation, Kalamazoo, MI, USA). During the inhalational challenge, the

animals were held in wire flow-through cage racks and filtered air was drawn through the 0.5-m³ inhalation chamber at a flow rate of 250 L/min. Animals were exposed to a low particle mass concentration of antigen, in the range 10–20 mg/m³ of aerosol, which was generated by controlled delivery of compressed air to a sidestream jet nebuliser (Trimed, Sydney, Australia) containing a solution of 2.5% ovalbumin in normal saline. The concentration and size distribution of particles within the breathing zone of the mice were continuously and cumulatively monitored using a light scatter device (PCAM-TX, PPM, Knoxville, TN, USA). In contrast to models involving uncontrolled exposure to high levels of aerosolized ovalbumin, this exposure protocol is not associated with the development of perivascular and peribronchiolar inflammation in the lung parenchyma but instead elicits lesions confined to the conducting airways. Control groups were nonsensitized nonexposed (i.e. naïve) wild-type and gene-targeted animals.

Airway reactivity

Responsiveness to methacholine was assessed in conscious, unrestrained mice by barometric plethysmography, using apparatus and software supplied by Buxco (Troy, NY, USA). This system yields a dimensionless parameter known as enhanced Pause (Penh), reflecting changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration, which can be used to empirically monitor airway function. Measurement was performed essentially as previously described [13]. Briefly, mice were placed in the chamber and baseline readings taken and averaged for 3 min. Aerosolized methacholine (concentrations in solution ranging from 3.125 to 50 mg/mL) was then delivered through an inlet into the chamber for 3 min and readings averaged over a period of 3 min after each dose was administered.

Cytokine production by peribronchial lymph node cells

This was assessed as described in detail elsewhere [14, 15]. Peribronchial lymph nodes (PBLN) were collected from sensitized, chronically exposed animals, and isolated cells were cultured in 100-µL mixed lymphocyte culture medium at 10⁶ cells/well in 96-well U-bottom microplates, either in the absence or the presence of 1 mg/mL of ovalbumin, for 72 h. Cell-free culture supernatants were stored at −70 °C and the content of IL-4, IL-5, IL-13 and IFN-γ subsequently determined by sandwich enzyme immunoassay.

Histopathology

Tissues for histopathological examination were collected 18 h after the last inhalational exposure and fixed in 10% buffered formalin overnight. The longitudinally orientated trachea and a horizontal slice from the mid-zone of the single-lobed left lung were embedded in paraffin. Ribbons of two to three sections cut at 5 µm were stained with haematoxylin and eosin for assessment of inflammatory changes in sections of the longitudinally orientated trachea; with Gordon & Sweet's reticulin stain for assessment of collagen deposition; or with Alcian blue-PAS for enumeration of mucin secreting cells in the intrapulmonary conducting airways.

Immunohistochemistry

To assess relative production of IgG₁ and IgG_{2a} by Ig-producing cells within the airway wall, immunostaining was performed using rabbit antibodies specific for each of these immunoglobulin subclasses (Zymed, San Francisco, CA, USA; 5 µg/mL) using a capillary action staining system as previously described [9]. Sections of formalin-fixed, paraffin-embedded tissue were subjected to antigen retrieval by boiling in 0.01 M citrate buffer (pH 6.0) for 10 min in a microwave. Sections were washed with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and non-specific binding was blocked with 50% normal goat serum for 10 min at 37 °C. They were then incubated for 30 min at 37 °C with an appropriate dilution of the primary antibody followed by a 1 : 50 dilution of swine anti-rabbit immunoglobulins and a 1 : 100 dilution of rabbit peroxidase-antiperoxidase complex (Dako, Sydney, Australia). Sections were washed four times with PBS-Triton after each incubation. Diaminobenzidine was used as the substrate and sections were counterstained with methyl green.

Morphometry

The validity and reliability of the morphometric techniques we employed for quantifying inflammation and remodelling have been established in previous reports [6–8]. To enumerate inflammatory cells and Ig-secreting cells, images were captured with a Spot Cooled Color Digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) which was calibrated with a reference measurement slide. Nuclear profiles in haematoxylin and eosin-stained sections, or numbers of cells exhibiting cytoplasmic immunoreactivity for immunoglobulins, were counted in every second consecutive microscopic field in the lamina propria, and the length of the epithelial basement membrane was measured by tracing using the Spot software. The data were used to calculate the mean number of cells per 100 µm of epithelial basement membrane in the lamina propria for individual animals.

In parallel, the full length of the epithelial surface was examined using a ×100 oil immersion objective to enumerate intraepithelial eosinophils, which were readily identified on the basis of their strongly staining cytoplasmic granules. The data were expressed as number of cells/mm of epithelial basement membrane for individual animals.

Reticulin-stained sections were used for assessment of subepithelial collagenization. The thickness of the reticulin-stained zone underneath the tracheal epithelium was measured and the mean thickness for each animal was calculated. The thickness of the epithelial layer was also measured in these sections and the mean thickness for each animal was calculated.

Metaplasia and/or hyperplasia of mucus-secreting goblet cells was assessed in sections of lung tissue stained with Alcian blue-PAS. The percentage of cells staining positive for mucins was enumerated in the largest visible airway and each animal was categorized according to a semilogarithmic scale as: grade 0 = <1% positive cells, grade 1 = 1–3%, grade 2 = 4–10%, grade 3 = 11–30% and grade 4 = ≥31%.

Statistical analysis

Results of morphometry and assessment of airway reactivity are presented as arithmetic mean ± standard error for each experimental group. For morphometric data, an initial one-way

analysis of variance followed by Newman–Keuls multiple comparison test was used to examine differences between groups. Comparison of airway reactivity to each dose of methacholine was by an unpaired *t*-test between groups of chronically exposed animals and the corresponding naïve control animals. Because measurements of the proportion of mucin-positive cells in the intrapulmonary airways were based upon grading, these results were expressed as median values and comparisons were by a non-parametric Mann–Whitney test. The software package GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for all data analysis and preparation of graphs.

Results

Airway inflammation

As previously described [6, 8], sensitized wild-type BALB/c mice exhibited accumulation of significant numbers of eosinophils within the epithelial layer following repeated exposure to aerosolized antigen. Similarly, accumulation of intraepithelial eosinophils was readily apparent in STAT6^{−/−} mice. The relative number of eosinophils within the epithelium was comparable in these two groups and was significantly increased compared to the corresponding naïve animals ($P < 0.001$ and $P < 0.01$, respectively; Fig. 1a). In contrast, accumulation of eosinophils was virtually abolished in IL-4/13 double-deficient mice.

In sensitized chronically exposed wild-type mice, there was a statistically significant increase in the mean profile density of cells in the airway wall as a consequence of accumulation of lymphocytes, plasma cells and other mononuclear inflammatory cells (Fig. 1b; $P < 0.001$ compared to naïve animals). Comparable accumulation of chronic inflammatory cells was also evident in the airways of both STAT6^{−/−} and IL-4/13^{−/−} mice ($P < 0.001$ for both).

Consistent with our previously reported observations in sensitized chronically exposed BALB/c mice [9], there were numerous cells immunoreactive for cytoplasmic IgG₁ in the lamina propria of the airways in wild-type mice (Fig. 2a) with almost no evidence of IgG_{2a}-positive cells (Fig. 2b). In contrast, in STAT6^{−/−} mice, there was a reduction in the number of IgG₁-positive cells (Fig. 2c) and a substantial increase in the number of IgG_{2a}-positive cells (Fig. 2d). Similar changes were observed in IL-4/13^{−/−} mice. The reduction in profile density of IgG₁-positive cells was significant ($P < 0.05$ for STAT6^{−/−} mice and $P < 0.01$ for IL-4/13^{−/−} mice) as was the increase in IgG_{2a}-positive cells ($P < 0.01$ for both; Fig. 3).

Subepithelial fibrosis

As previously described, a prominent subepithelial reticulin-stained zone was apparent in sensitized wild-type mice exposed to antigen. Compared to control animals, the mean thickness of this zone was increased approximately 1.5-fold ($P < 0.001$; Fig. 4a). Equivalent subepithelial collagenization was observed in STAT6^{−/−} mice. In naïve IL-4/13^{−/−} mice, the thickness of the subepithelial zone was markedly diminished relative to wild-type mice ($P < 0.001$). As a result, whereas the subepithelial layer of collagen in IL-4/13^{−/−} mice was significantly increased compared to the corresponding control animals, the thickness

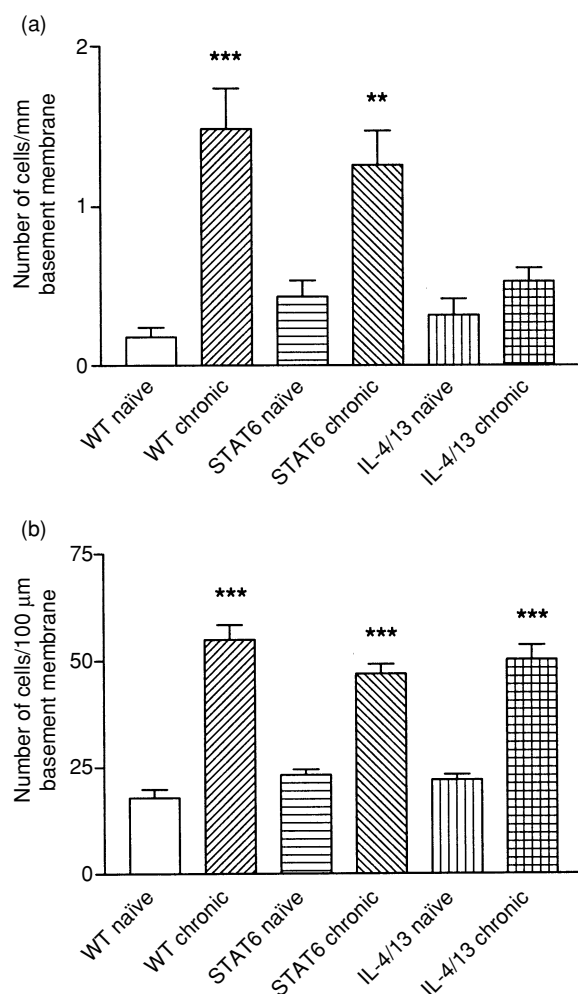


Fig. 1. (a) Intraepithelial eosinophils and (b) inflammatory cells in the lamina propria of the trachea of naïve and sensitized, chronically exposed wild-type, STAT6^{-/-} and IL-4/13^{-/-} mice. Significant differences compared to corresponding naïve animals are shown as ** $P < 0.01$, *** $P < 0.001$ (for all morphometric assessment, $n = 6$ for wild-type naïve and $n = 7$ for STAT6^{-/-} naïve animals, $n = 8$ for all other groups).

of this layer was essentially the same as that in wild-type naïve mice (Fig. 4a).

Epithelial changes

The mean thickness of the airway epithelium was significantly increased in sensitized wild-type BALB/c mice after 6 weeks of inhalational exposure to antigen ($P < 0.01$ compared to naïve control animals; Fig. 4b). Again, comparable changes were noted in STAT6^{-/-} mice, whereas in IL-4/13^{-/-} mice the epithelial thickness was essentially the same as in wild-type naïve mice although it was significantly increased compared to the corresponding control animals.

Mucus-secreting goblet cells were virtually absent in the intrapulmonary airways of naïve wild-type mice and this was also true of naïve STAT6^{-/-} and IL-4/13^{-/-} animals, so that on morphometric quantification the median grade recorded for these groups was zero. Consistent with our previous reported studies, there was a striking increase in the number of goblet cells in the intrapulmonary airways of sensitized exposed wild-type BALB/c mice, with a median grade of 3 (interquartile range

3–4). Few goblet cells were observed in chronically exposed STAT6^{-/-} and IL-4/13^{-/-} mice and the median grades for these animals were 1 (0.5–1.5) and 0.5 (0–1), respectively. These differences were statistically significant ($P < 0.01$ in the case of STAT6^{-/-} mice and $P < 0.001$ in the case of IL-4/13^{-/-} mice).

Airway responsiveness

Baseline absolute Penh for all groups of mice was virtually identical, with individual animals all exhibiting values in the range 0.29–0.41. As reported previously, exposure to increasing concentrations of aerosolized methacholine increased Penh values in naïve wild-type mice [7, 8]. The increase was approximately fivefold relative to baseline at a concentration of 50 mg/mL (Fig. 5a). Sensitized chronically exposed wild-type animals exhibited a left-shifted dose–response curve and increased maximal reactivity, characteristic of AHR (Fig. 5a). Compared to controls, the increase in Penh was significantly greater at all methacholine concentrations above 3.125 mg/mL. Naïve STAT6^{-/-} mice were similar to naïve wild-type mice in their response to methacholine, with a threefold increase in Penh at 50 mg/mL compared to baseline, while sensitized chronically exposed STAT6^{-/-} mice had an even further left-shifted dose–response curve, with a maximal response achieved at a methacholine concentration of 12.5 mg/mL (Fig. 5b). In contrast, naïve IL-4/13^{-/-} mice exhibited much greater responsiveness to methacholine than naïve wild-type animals, with a greater than 10-fold increase in Penh relative to baseline at 50 mg/mL. These animals did not exhibit any evidence of hyper-reactivity following sensitization and chronic exposure (Fig. 5c).

Cytokine production by peribronchial lymph node cells

PBLN cells from sensitized chronically exposed wild-type mice secreted low levels of IL-5, IL-13 and IFN- γ in the absence of *in vitro* restimulation with ovalbumin. In the presence of ovalbumin, IL-4 was also detectable in the culture supernatants and concentrations of the other cytokines in the culture supernatants were markedly increased (Table 1). In contrast, PBLN cells from STAT6^{-/-} mice did not secrete detectable IL-4 even after restimulation with ovalbumin and secreted lower levels of IL-5 and IL-13, but higher levels of IFN- γ ($P < 0.01$ for all comparisons). PBLN from IL-4/13^{-/-} mice did not secrete any detectable IL-4 or IL-13, but like the STAT6^{-/-} mice secreted lower levels of IL-5 and higher levels of IFN- γ ($P < 0.01$).

Discussion

In this study, we assessed the role of the prototypic Th2 cytokines IL-4 and IL-13 in the development of airway inflammation, remodelling and hyper-reactivity in a mouse model of chronic asthma. We compared responses in wild-type mice with those in mice lacking both IL-4 and IL-13, as well as with mice lacking STAT6, a key pathway for signalling by these Th2 cytokines. Among the major findings of this study were that whereas IL-4/13 double-deficient mice exhibited little if any recruitment of eosinophils into the airway epithelium and no evidence of AHR to methacholine, both inflammation and AHR were undiminished in STAT6-deficient mice.

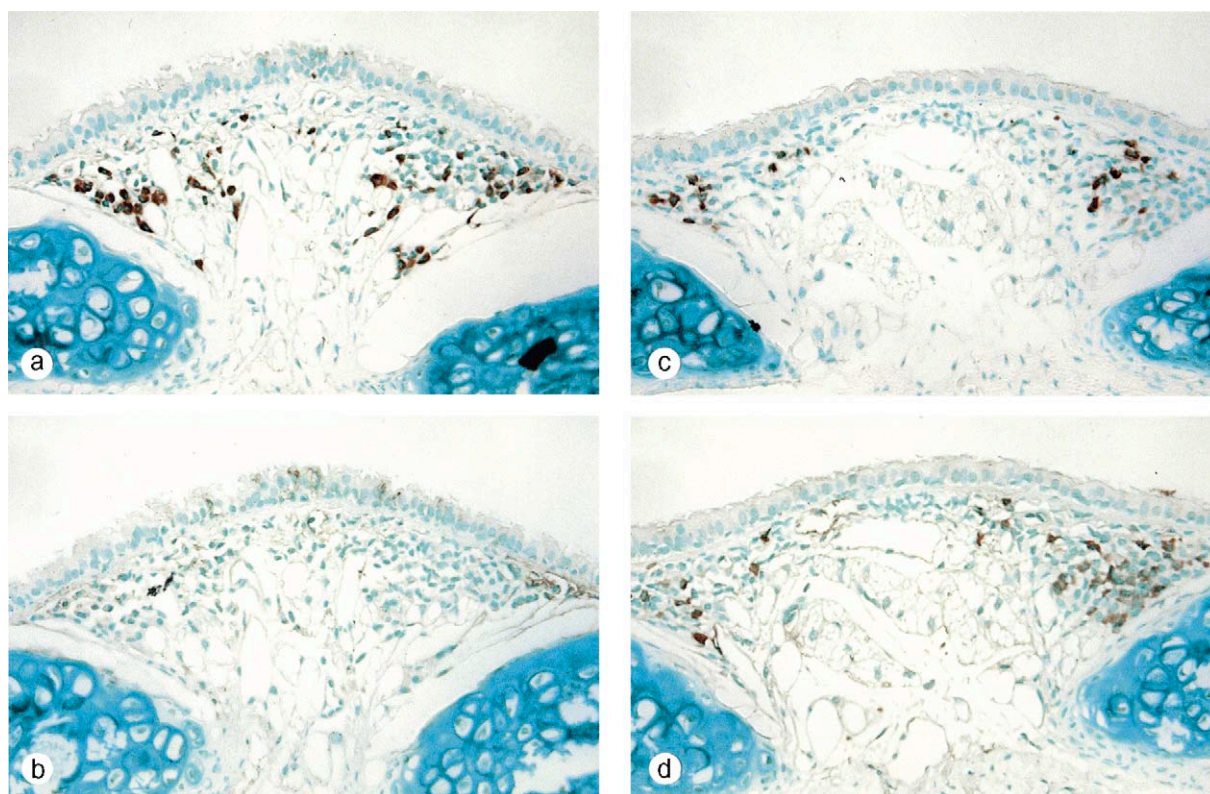


Fig. 2. IgG₁-immunoreactive (a, c) and IgG_{2a}-immunoreactive (b, d) plasma cells in the lamina propria of sensitized, chronically exposed wild-type (a, b) and STAT6^{-/-} (c, d) mice.

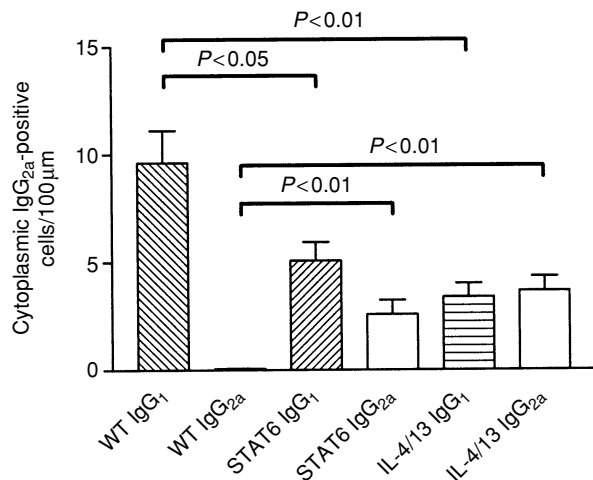


Fig. 3. IgG₁- and IgG_{2a}-immunoreactive cells in the lamina propria of sensitized, chronically exposed wild-type, STAT6^{-/-} and IL-4/13^{-/-} mice. Significant differences between groups are as indicated.

The latter results are in marked contrast to observations in STAT6^{-/-} mice in short-term, high-level exposure models of allergic bronchopulmonary inflammation [16–20]. In those studies, a variable degree of inhibition of eosinophil recruitment was reported, with differences possibly related to the mouse strain or the inhalation exposure protocol employed. AHR consistently failed to develop in the STAT6^{-/-} mice following short-term exposure to antigen. Similarly, recent studies involving adoptive transfer experiments have emphasized

STAT6 dependence of the development of AHR in such models [21, 22].

In this model of chronic asthma, neither the airway inflammatory response nor the development of AHR was inhibited in STAT6-deficient animals, implying that alternative mechanisms of cytokine signalling may be activated/induced following chronic antigenic challenge. These results are broadly in agreement with those of Blease et al. who recently demonstrated development of AHR in STAT6^{-/-} mice in a model of chronic fungal asthma [23]. Their experimental system involves intrapulmonary deposition of persistent fungal conidia, rather than multiple inhalational challenges with soluble antigen, and is associated with marked parenchymal inflammation progressing to pulmonary fibrosis, which complicates interpretation of the data. In contrast, in our model, inflammation is confined to the airway wall and the observation that plasma cells in the airways were essentially all IgG₁-positive and IgG_{2a}-negative is indicative of a local Th2 pattern of response [24]. The shift towards IgG_{2a} synthesis in both STAT6- and IL-4/13-deficient mice is consistent with the expected abrogation of the Th2 response in these animals, as is the altered profile of cytokines produced by PBLN.

Taken together with our previously reported investigations of the effects of deficiencies of IL-4, IL-5, IL-13 and IL-4R α [7, 8], the present study provides new insights into the relative contribution of Th2 cytokines and their signalling pathways in the pathogenesis of lesions of chronic asthma. With respect to the inflammatory response, gene-targeted animals deficient in either IL-5 and IL-13 exhibited little if any accumulation of intraepithelial eosinophils [7, 8], while the present investigation

demonstrates that this is also true of animals deficient in both IL-4 and IL-13. In contrast, numbers of these cells were un-diminished in mice that were deficient in IL-4 [7], the IL-4R α chain which is common to receptors for both IL-4 and IL-13 [8], or (as demonstrated in this study) the STAT6-signalling pathway which is utilized by both IL-4 and IL-13 [11]. These data are consistent with our previous suggestion that, in chronic asthma, IL-13 signalling may be able to occur via mechanisms distinct from those also utilized by IL-4 [8]. The findings are supported

by the remarkably similar picture with respect to accumulation of chronic inflammatory cells in the lamina propria of the airways.

The improved mouse model of chronic asthma that we have developed offers opportunities for investigation of the mechanisms of airway wall remodelling, which is not feasible using

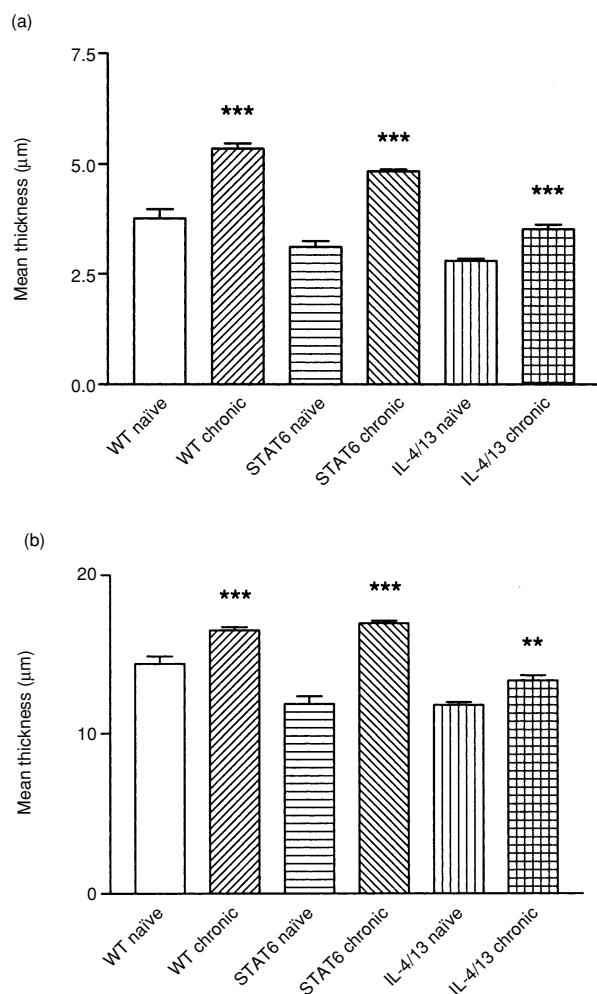


Fig. 4. (a) Subepithelial collagenization and (b) hypertrophy of the tracheal epithelium in naïve and sensitized, chronically exposed wild-type, STAT6 $^{-/-}$ and IL-4/13 $^{-/-}$ mice. Significant differences compared to corresponding naïve animals are shown as ** P < 0.01, *** P < 0.001.

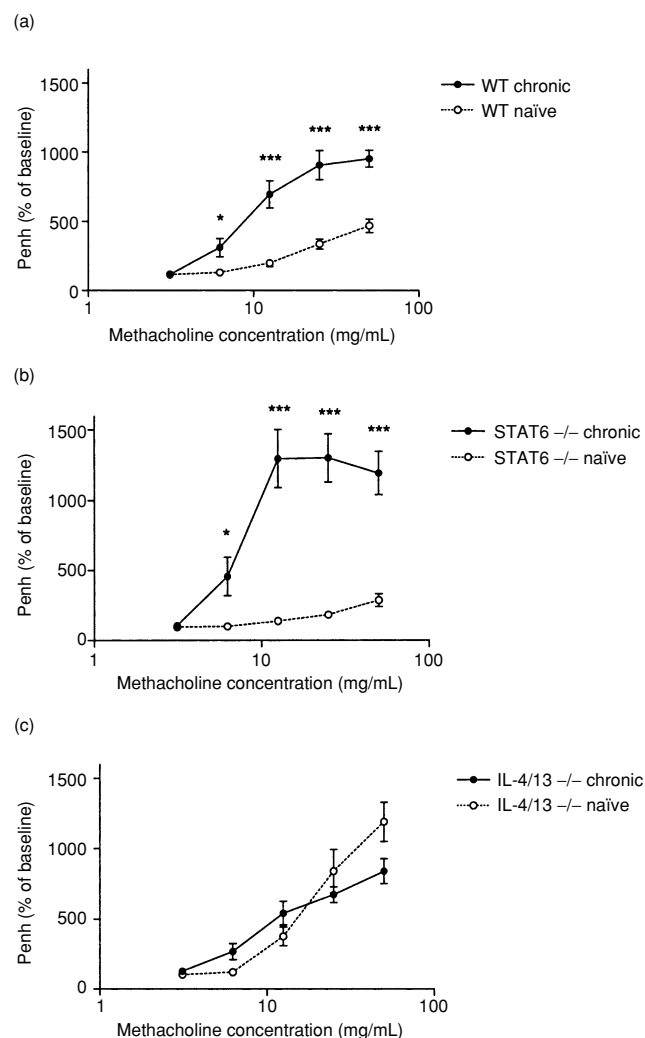


Fig. 5. Airway reactivity of naïve and sensitized chronically exposed mice, assessed by change in Penh in response to increasing concentrations of aerosolized β -methacholine. (a) Wild-type mice (●, chronic; ○, naïve); (b) STAT6 $^{-/-}$ mice (●, chronic; ○, naïve); (c) IL-4/13 $^{-/-}$ mice (●, chronic; ○, naïve). Significant differences compared to naïve animals are shown as * P < 0.05, *** P < 0.001 (n = 7 for wild-type naïve and chronically challenged animals, n = 8 for all other groups).

Table 1. Cytokine production by peribronchial lymph node cells

Cytokine	– OVA <i>in vitro</i>			+ OVA <i>in vitro</i>		
	Wild-type	STAT6 $^{-/-}$	IL-4/13 $^{-/-}$	Wild-type	STAT6 $^{-/-}$	IL-4/13 $^{-/-}$
IL-4	ND	ND	ND	0.10 $\pm$ 0.02	ND	ND
IL-5	1.65 $\pm$ 0.05	2.19 $\pm$ 0.15	0.67 $\pm$ 0.02*	3.20 $\pm$ 0.13	1.39 $\pm$ 0.05*	1.01 $\pm$ 0.03*
IL-13	0.54 $\pm$ 0.04	0.49 $\pm$ 0.08	ND	2.22 $\pm$ 0.16	0.54 $\pm$ 0.04*	ND
IFN- γ	1.15 $\pm$ 0.10	1.70 $\pm$ 0.18	2.90 $\pm$ 0.06*	14.60 $\pm$ 0.86	29.06 $\pm$ 1.03*	26.47 $\pm$ 2.28*

Expressed as ng/mL of 72-h supernatants of cells cultured at 10^6 /well. *Significantly different compared to wild-type mice, P < 0.01. ND, not detectable.

short-term models. The magnitude of the increased thickness of the subepithelial collagenous layer observed in these animals is similar to that reported in human studies (e.g. [25, 26]). We previously showed that both subepithelial fibrosis and epithelial hypertrophy are dependent on IL-13, because neither of these changes develops in IL-13^{-/-} animals [8]. In contrast, IL-4-deficient animals exhibit enhanced subepithelial fibrosis and epithelial hypertrophy compared to wild-type mice [7]. The effect of IL-13 on subepithelial fibrosis is apparently not dependent on IL-4R α -mediated signalling [8] and the present study indicates that it is also not dependent on signalling via STAT6. The situation is more complicated with respect to epithelial thickening, which does appear to be IL-4R α -dependent [8]. An intriguing finding of the present investigation is that in IL-4/13 double-deficient animals, the effect of IL-4 deficiency appears to dominate, because these mice exhibit significant subepithelial fibrosis and epithelial hypertrophy relative to their naïve controls. However, for reasons that remain unclear, these naïve mice have thinner epithelium and a thinner subepithelial reticulin-stained zone than naïve wild-type mice, so that the absolute magnitude of the responses of chronically challenged IL-4/13 double-deficient animals is not significantly different from control wild-type mice. Consistent with data from studies of short-term models of allergic bronchopulmonary inflammation, mucous cell hyperplasia/metaplasia was absent in animals deficient in IL-13 or the IL-4R α chain [8] as well as in STAT6-deficient and IL-4/13 double-deficient mice (this study).

With respect to AHR, the present investigation emphasizes the complex, integrated interplay between Th2 cytokines in the pathogenesis of this functional lesion. We have previously established that AHR is completely absent in IL-5^{-/-} mice in this model of chronic asthma, but is exaggerated in IL-4-deficient mice [7] and persists in IL-13-deficient animals even in the absence of intraepithelial accumulation of eosinophils or of remodelling [8]. On the basis of the results of the present study, it appears that induction of AHR requires the presence of either IL-13 or IL-4, because AHR does not develop in double-deficient mice. It is worth noting that naïve animals deficient in either IL-13 alone [8] or both IL-4 and IL-13 (this study) exhibit an exaggerated increase in Penh in response to methacholine. This is presumably related to the IL-13 deficiency, which may be associated with induction of other as yet undefined cytokine pathways that could stimulate AHR, although the augmented response in naïve animals does not prevent the development of AHR in IL-13^{-/-} mice [8].

The results of this study are consistent with our previous observations in a short-term model in which AHR in IL-13-deficient mice was abolished if the animals were treated with an antibody to IL-4 [15]. In the chronic exposure model, neither signalling via receptor complexes involving the IL-4R α chain [8] nor activation of the STAT6 pathway (this study) is necessary for development of AHR. However, other populations of cells and their mediators may interact with Th2 cytokines and contribute to the pathogenesis of AHR. For example, Th1 cells or cells of intermediate differentiation may be able to regulate the development of AHR [27, 28], possibly via cytokines such as IFN- γ [29–31]. It is noteworthy, however, that the profile of cytokine production by peribronchial lymph node cells from STAT6^{-/-} and IL-4/13^{-/-} mice was remarkably similar, so that additional as yet undefined mediators must be responsible for

the marked phenotypic differences between these strains following chronic inhalational challenge.

In summary, the results of this study reinforce the concept that the pathobiology of chronic asthma is significantly different from that revealed by the study of models of acute allergic bronchopulmonary inflammation [5]. Furthermore, they imply that while Th2 cytokines play key roles in chronic asthma, inhibition of their signalling pathways is less likely to be therapeutically useful than inhibition of the cytokines themselves. In this context, it is of interest that persisting inflammation and AHR associated with induction of chronic fungal allergic inflammation in STAT6^{-/-} mice could be inhibited by treatment with a toxic IL-13 fusion protein [23]. Nevertheless, recent clinical trials [32] emphasize the redundancy of cytokine activities and the complexity of the inflammatory response in asthma, suggesting that targeting single molecules or pathways may be a poor therapeutic strategy.

Acknowledgements

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