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Role of STAT6 and SMAD2 in a model of chronic allergen exposure: a mouse strain comparison study

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Clinical and Experimental Allergy

Summary

Background Asthma is a disease characterized by variable and reversible airway obstruction and is associated with airway inflammation, airway remodelling (including goblet cell hyperplasia, increased collagen deposition and increased smooth muscle mass) and increased airway responsiveness. It is believed that airway inflammation plays a critical role in the development of airway remodelling, with IL-13 and TGF- β 1 pathways being strongly associated with the disease progression. Mouse models of asthma are capable of recapitulating some components of asthma and have been used to look at both IL-13 and TGF- β 1 pathways, which use STAT6 and SMAD2 signalling molecules, respectively.

Objectives Using brief and chronic models of allergen exposure, we utilized BALB/c and C57Bl/6 to explore the hypothesis that observed differences in responses to allergen between these mouse strains will involve fundamental differences in IL-13 and TGF- β 1 responses.

Methods The following outcome measurements were performed: airway physiology, bronchoalveolar lavage cell counts/cytokine analysis, histology, immunoblots and gene expression assays.

Results We demonstrate in BALB/c mice an IL-13-dependent phosphorylation of STAT6, nuclear localized in inflammatory cells, which is associated with indices of airway remodelling and development of airway dysfunction. In BALB/c mice, phosphorylation of SMAD2 is delayed relative to STAT6 activation and also involves an IL-13-dependent mechanism. In contrast, despite an allergen-induced increase in IL-4, IL-13 and eosinophils, C57Bl/6 demonstrates a reduced and distinct pattern of phosphorylated STAT6, no SMAD2 phosphorylation changes and fail to develop indices of remodelling or changes in airway function.

Conclusion The activation of signalling pathways and nuclear translocation of signalling molecules downstream of IL-13 and TGF- β 1 further support the central role of these molecules in the pathology and dysfunction in animal models of asthma. Activation of signalling pathways downstream from IL-13 and TGF- β 1 may be more relevant in disease progression than elevations in airway inflammation alone.

Keywords airway remodelling, asthma, Th1/Th2 cells, transgenic/knockout mice

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Introduction

Asthma is a disease characterized by variable and reversible airway obstruction and is associated with airway inflammation, airway remodelling and airway hyper-responsiveness (AHR). While the precise mechanisms are poorly understood, it is believed that AHR in asthma is

secondary to both inflammatory and remodelling changes in the airways [1]. It is further believed that airway inflammation plays a critical role in the development of airway remodelling, although the relative contribution and temporal actions of various mediators or cells (e.g. IL-13, TGF- β 1, eosinophils) remain to be determined. Furthermore, with currently available therapies more

efficacious in treating the inflammation as compared with established airway remodelling, there remains a need to understand the pathways involved in the development and maintenance of the remodelled airway.

IL-13 is a central mediator in allergic asthma and has been implicated in numerous ways with the development of pathology and functional changes [2]. The pleiotropic nature of IL-13 with respect to the development of goblet cell hyperplasia [2, 3], increased responsiveness of smooth muscle [4] and induction of the pro-fibrotic mediator TGF- β 1 [5] make it a promising target for therapies. We and others have demonstrated that IL-13 is crucial for the development of several indices of airway remodelling and the associated sustained AHR following chronic allergen exposure in mice [2, 3]. Based on this pre-clinical evidence, there is much interest in IL-13 signalling as a site of intervention in the treatment of asthma.

TGF- β 1 is also increased in asthmatic airways and has been positively correlated with basement membrane thickness in bronchial mucosal biopsies [6]. Additionally, it has been demonstrated that in bronchial epithelial cells, differential expression of TGF- β 1 is observed in asthmatics as compared with normal controls [7]. In asthmatic airways, it is believed that TGF- β 1 may play dual roles as an anti-inflammatory [7] and a wound-healing mediator [8]. In airway disease models, TGF- β 1 has been directly implicated in fibrosis [9] and is involved in the fibrotic remodelling observed in mouse models of chronic allergen exposure [10].

Recent evidence suggests that IL-13 signalling through the IL-13 R α 2 chain up-regulates TGF- β 1, implying that IL-13 actions may precede those of TGF- β 1 [11]. Indeed, Elias *et al.* have previously demonstrated that in IL-13 transgenic mice, IL-13 mediated up-regulation of active TGF- β 1, and this was associated with airway fibrosis [5].

Given the evidence that supports both IL-13 and TGF- β 1 in the development of airway remodelling and associated AHR, we sought to investigate their roles in a murine model of allergen exposure. We and others have observed that remodeling and airway AHR varies

markedly between mouse strains [12]. Specifically, we have observed that BALB/c, but not C57Bl/6 mice, develop AHR following brief or chronic allergen exposure. Here, we explore the hypothesis that the difference in responses to allergen between these mouse strains will involve fundamental differences in IL-13 and TGF- β 1 responses. To test this hypothesis, we looked at brief and chronic allergen exposure at multiple time-points in two genetically distinct, commonly used inbred mouse strains, BALB/c and C57Bl/6.

Methods

Animals

Female BALB/c and C57Bl/6 wild-type mice, aged 8–10 weeks, were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). Female mice of a BALB/c background and similar age, deficient in IL-4 (IL-4^{-/-}) or IL-13 (IL-13^{-/-}) were obtained from the Jackson Laboratory or kindly provided by Dr Andrew McKenzie at the Medical Research Council Laboratory of Molecular Biology, Cambridge, UK, respectively. 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 McMaster University, and conformed to the NIH guidelines for experimental use of animals.

Sensitization and exposure

Mice were sensitized as described previously [13]. Briefly, all mice received intraperitoneal (IP) injections of ovalbumin (OVA) conjugated to aluminium potassium sulphate on Days 1 and 11 and intranasal (IN) OVA on Day 11. Following sensitization, mice were subjected to an allergen exposure protocol with multiple time-points for outcome measurements (Fig. 1). Brief allergen exposure was composed of a single 2-day period of intranasal

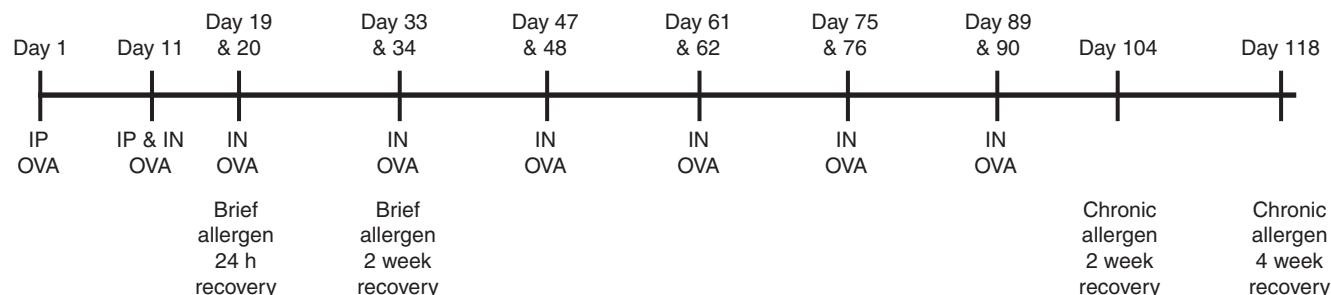


Fig. 1. Allergen sensitization and exposure protocol. Mice were sensitized with intraperitoneal ovalbumin (IP OVA) injections on Days 1 and 11 and intranasal (IN) OVA on Day 11. Brief allergen exposure comprised a single 2-day period of IN OVA administration (100 μ g in 25 μ L saline). Chronic allergen exposure comprised six 2-day periods of IN OVA administration, each separated by 12 days. Exposures started on Days 19 and 20. Control animals received saline instead of OVA.

ovalbumin (IN OVA) administration (100 µg in 25 µL saline) under gaseous anaesthesia, with ventilation rate visually monitored between strains to ensure equal plane of anaesthesia before IN exposure. Chronic allergen exposure was composed of six 2-day periods of IN OVA administration, each separated by 12 days. Exposures started on Days 19 and 20.

Outcome measurements were made at the following time-points; (i) 24 h after a single 2-day period of allergen exposure (Day 21), (ii) 2 weeks following a single 2-day period of allergen exposure (Day 34), (iii) 2 weeks following the complete chronic allergen exposure protocol (Day 104) and (iv) 4 weeks following the complete chronic allergen exposure (Day 118).

Airway responsiveness

Airway responsiveness was measured by total respiratory system resistance (R_{RS}) responses to intravenous saline and increasing doses of methacholine (MCh) using the Flow Interrupter ventilator system ($n=8$ per group) [13]. Airway physiology measurements were made on mice 24 h after a single period of allergen exposure and 4 weeks following the chronic allergen exposure protocol.

Bronchoalveolar lavage and serum analysis

Bronchoalveolar lavage (BAL) cell differential analysis was performed as previously described [13]. Blood was collected by cardiac puncture. ELISAs were performed for IL-4, IL-13, IFN- γ and active TGF- β 1 in BAL supernatant and IgE levels in serum (BD Biosciences, San Diego, CA, USA, and R&D Systems, Minneapolis, MN, USA).

Lung histology

Lungs were dissected and left lobes inflated with 10% formalin with a pressure of 25 cm H₂O, ligated at the trachea, and fixed in 10% formalin for 24 h. Three-micron-thick sections were stained with Mayer's haematoxylin/eosin (H&E), periodic acid-Schiff (PAS) and picrosirius red (PSR) for assessing the presence of tissue eosinophils, goblet cells and collagen, respectively. Sections were immunostained using antibodies against α -smooth muscle actin (α -SMA) (Dako, Denmark), phospho-SMAD2 (pSMAD2) (Cell Signaling Technology Inc., Danvers, MA, USA) and phospho-STAT6 (pSTAT6) (Abcam, Cambridge, MA, USA). All immunostains required blockage of endogenous peroxidase with a 1.5% H₂O₂/methanol solution for 30 min before 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. α -SMA, pSMAD2 and pSTAT6 primary antibodies were

diluted at 1:100, 1:1000 and 1:100, respectively, and incubated overnight at 4 °C. The next morning extensive washes with Tris buffer (pH 7.6) were performed to remove primary antibodies. In between each addition of reagent, extensive washes with Tris were performed with squeeze bottles. 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 into 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.

Lung morphometry

PAS, PSR and α -SMA-stained tissue sections were viewed and images collected under 20 $\times$ objective magnification light microscopy (Olympus BX40; Carsen Group Inc., Markham, ON, Canada). For quantifying the stain, a customized digital image analysis system (Northern Eclipse, Version 7.0; Empix Imaging Inc., Mississauga, ON, Canada) with an attached digital pen and drawing tablet was used to collect and analyse images as described previously [14]. H&E-stained tissue sections were viewed and images collected under 40 $\times$ objective. Eosinophils were expressed as number/area in a 50 µm band extending from the basement membrane of the large primary bronchus as previously described [13]. pSMAD2- and pSTAT6-stained tissue sections were viewed and collected under 40 $\times$ objective. Quantification of pSMAD2 and pSTAT6 positively stained cells was expressed as the percentage of positively stained cells (positive cells/total cells $\times$ 100).

Immunoblots

See details in the Supporting information.

Gene expression analysis

See details in the Supporting information.

Lung deposition of intranasal solutions in BALB/c and C57Bl/6 assessed by positron emission tomography/computed tomography

See details in the Supporting information.

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 were made using Student's *t*-tests, assuming equal variances.

Differences were assumed to be statistically different when the observed *P*-values were less than 0.05.

Results

BALB/c and C57Bl/6 inflammatory profiles following brief allergen exposure

Consistent with the development of an allergic phenotype, both BALB/c and C57Bl/6 mice demonstrated significant increases in serum IgE levels following sensitization and brief exposure to OVA ($P < 0.05$; Supporting information Fig. S1a). Allergen-induced elevations of the Th2 cytokines, IL-4 and IL-13, were detected in BAL fluid following brief allergen exposure in both BALB/c and C57Bl/6 mice ($P < 0.05$; Supporting information Fig. S1b, c). No detectable changes in the Th1 cytokine, IFN- γ , were observed in the BAL fluid for either strain of mice (Supporting information Fig. S1d). Increases in BAL and tissue eosinophils were also observed in both BALB/c and C57Bl/6 mice ($P < 0.05$; Supporting information Fig. S1e, f). Active levels of TGF- β were assessed by ELISA and were below detection levels in both strains of mice (data not shown). For each inflammatory mediator/marker assessed, BALB/c mice demonstrated a trend for a greater response as compared with C57Bl/6 mice, although this was not statistically significant for any mediator assessed.

Lung deposition of intranasal solutions in BALB/c and C57Bl/6 assessed by positron emission tomography/computed tomography

To confirm that our observed strain-dependent results were the product of similar distribution patterns of IN OVA, we performed *in vivo* imaging of IN 18-fluorodeoxyglucose (18-FDG) using PET/CT administered in identical fashion to OVA. We performed *in vivo* whole-body PET/CT imaging immediately after intranasal instillation of 25 μ L of 18-FDG (Fig. 2). The mean percentage of total radioactivity (adjusted for amount of radioactivity delivered and body weight) in BALB/c mice was 42.25% with standard deviation (SD) of 11.19%, while in C57Bl/6 mice, the detected radioactivity was 32.29% with SD of 16.64%. No statistically significant difference in the distribution of IN 18-FDG was observed ($P > 0.05$, $n = 5$ for each group). To detect statistical significance, a sample size of 31 would be required based on the means and SDs obtained from this group of animals.

Activation of STAT6 and SMAD2 pathways following brief allergen exposure

We and others have shown that IL-4 and IL-13 are necessary for development of AHR and indices of airway

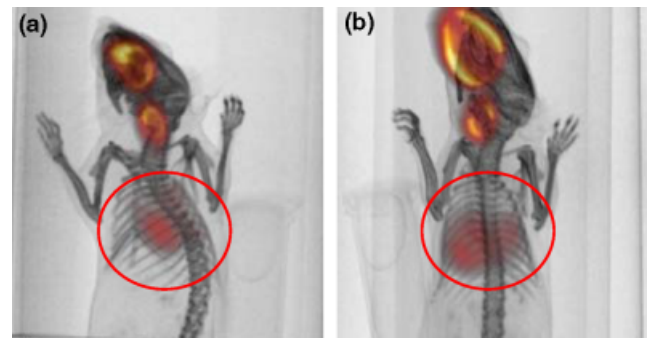


Fig. 2. Representative images from small animal whole-body positron emission tomography/computed tomography imaging of BALB/c and C57Bl/6 mice following intranasal administration of 25 μ L of 18-fluorodeoxyglucose (18-FDG). Red circles outline distribution of 18-FDG to the lung. (a) BALB/c mice; (b) C57Bl/6 mice.

remodelling [2, 3, 15]. Here, we determined the activity of the common signalling pathway of IL-4 and IL-13 by investigating the phosphorylation of STAT6 (Fig. 3a–g). Morphology and morphometry were used for qualitative and quantitative assessment of pSTAT6, respectively.

We observed allergen-induced increases in BAL IL-4 and IL-13 in both BALB/c and C57Bl/6 mice, with a greater response in the former strain. Despite elevations in IL-4 and IL-13 observed in both strains, activation of the STAT6 pathway, as indicated by pSTAT6 staining, was observed 24 h following brief allergen exposure in BALB/c, but not C57Bl/6 mice ($P < 0.05$; Fig. 3a). The increased BALB/c pSTAT6 staining was nuclear localized in small, nuclear dense, inflammatory cells with lymphocyte morphology ($P < 0.05$; Fig. 3c).

The activation of the STAT6 pathway in BALB/c mice was sustained as evidenced by significant increases in pSTAT6-stained positive cells 2 weeks following brief allergen exposure ($P < 0.05$; Fig. 3a). Consistent with the data obtained at the earlier time-point, positive-staining cells 2 weeks after allergen exposure in BALB/c mice demonstrated nuclear localization of pSTAT6 in small, nuclear dense, inflammatory cells with lymphocyte morphology (Fig. 3d).

C57Bl/6 mice demonstrated no significant increases in pSTAT6 staining at either the 24-h or 2-week time-points ($P < 0.05$; Fig. 3a), although some staining was observed in both control and allergen-exposed animals that appeared to be membrane localized in large, single-nucleated, inflammatory cells with macrophage morphology (Fig. 3e and g).

TGF- β 1 mRNA or active protein levels are not absolute markers of TGF- β 1 activation [16]. Recently, it has been suggested that SMAD2 phosphorylation is a more relevant measure of TGF- β 1 activation [17, 18]. We therefore determined the activity of the TGF- β 1 pathway by investigating the phosphorylation of SMAD2 (Fig. 3h).

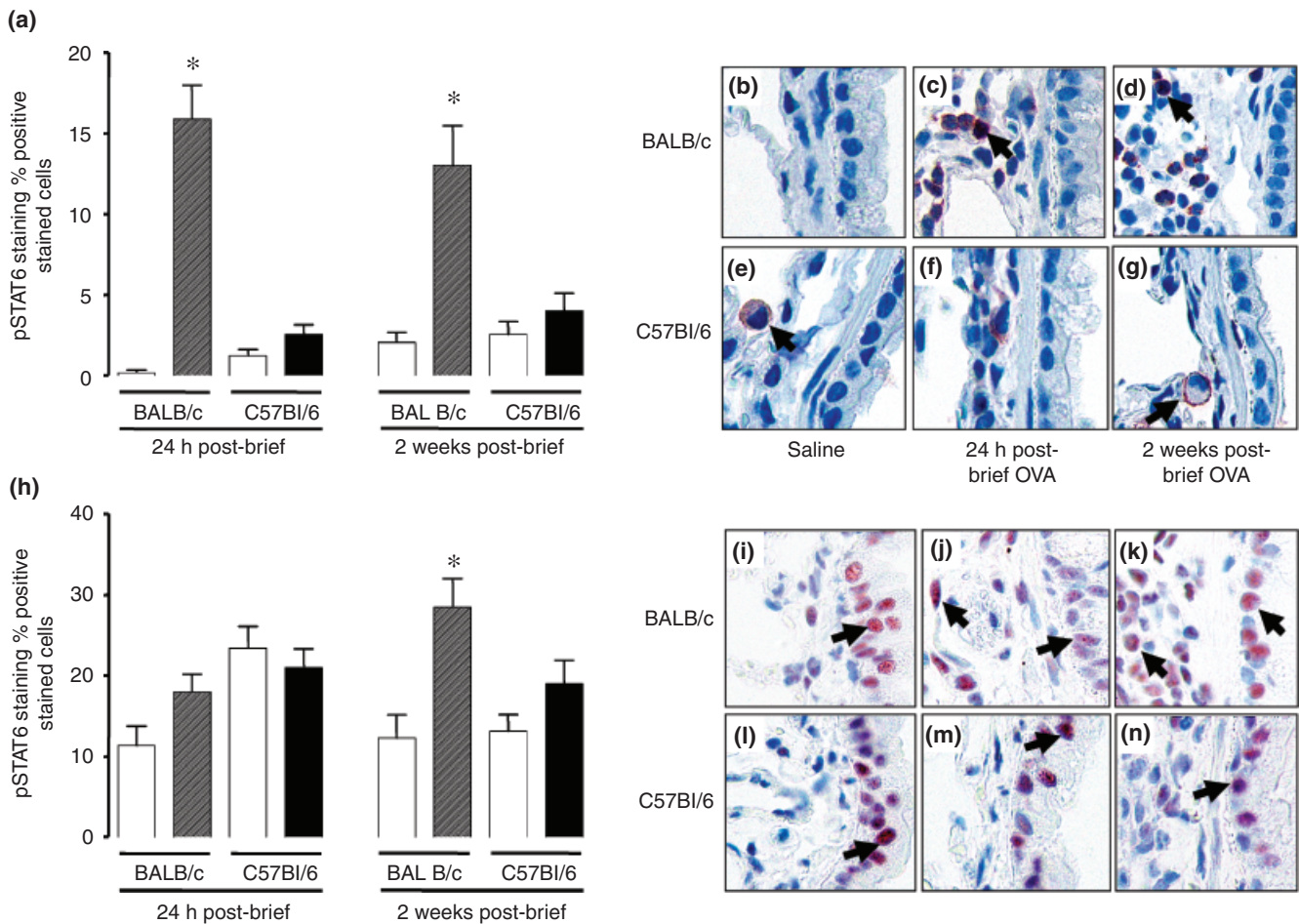


Fig. 3. STAT6 and SMAD2 phosphorylation following brief allergen exposure. (a) Morphometric analysis of pSTAT6 staining in mice following brief allergen exposure (positive-stained cells/total cell number $\times 100$). (b–g) Representative samples of pSTAT6 staining following brief allergen exposure: (b) BALB/c brief saline exposed; (c) BALB/c brief ovalbumin (OVA) exposed – 24-h recovery period; (d) BALB/c brief OVA exposed – 2-week recovery period; (e) C57Bl/6 brief saline exposed; (f) C57Bl/6 brief OVA exposed – 24-h recovery; (g) C57Bl/6 brief OVA exposed – 2-week recovery; (h) morphometric analysis of pSMAD2 staining in mice following brief allergen exposure (positive-stained cells/total cell number $\times 100$). (i–n) Representative samples of pSMAD2 staining following brief allergen exposure: (i) BALB/c brief saline exposed; (j) BALB/c brief OVA exposed – 24-h recovery period; (k) BALB/c brief OVA exposed – 2-week recovery period; (l) C57Bl/6 brief saline exposed; (m) C57Bl/6 brief OVA exposed – 24-h recovery; (n) C57Bl/6 brief OVA exposed – 2-week recovery. BALB/c saline: open white bar; BALB/c OVA: hatched white bar; C57Bl/6 saline: open white bar; C57Bl/6 OVA: solid black bar. Data are expressed as mean (SEM); 10 mice per group. * indicates significant difference from corresponding control animals ($P < 0.05$).

Immediately following brief allergen exposure, no detectable increases in pSMAD2 levels were observed in BALB/c or C57Bl/6 mice (Fig. 3h). A delayed elevation in nuclear-localized pSMAD2 levels was observed 2 weeks following brief allergen exposure in BALB/c, but not C57Bl/6 mice (Fig. 3k). The pSMAD2 staining in BALB/c mice was apparent in epithelial, smooth muscle and inflammatory cells.

To support and confirm our immunohistochemistry of pSTAT6 staining, we performed immunoblot analysis on tissues from BALB/c and C57Bl/6 mice 24 h following brief allergen exposure (Fig. 4a–c). We demonstrate in whole lung homogenate that pSTAT6 is elevated following allergen exposure in BALB/c mice, but not C57Bl/6 mice. No allergen-induced differences in total STAT6

expression were observed in either BALB/c or C57Bl/6 mice (data not shown).

It has been demonstrated in transgenic mice that TGF- β 1 gene expression and production may be downstream of IL-13 signalling [5]. We explored this hypothesis and demonstrate that in BALB/c mice, pSTAT6 signalling is associated with TGF- β 1 gene expression ($P < 0.05$; Supporting information, Fig. S2). In C57Bl/6 mice, reduced and distinct patterns of pSTAT6 staining are associated with no changes in TGF- β 1 gene production. Finally, the elevation in pSMAD2 levels in BALB/c mice ($P < 0.05$; Fig. 3h) was consistent with TGF- β mRNA transcript levels that were significantly increased ($P < 0.05$; Supporting information Fig. S2).

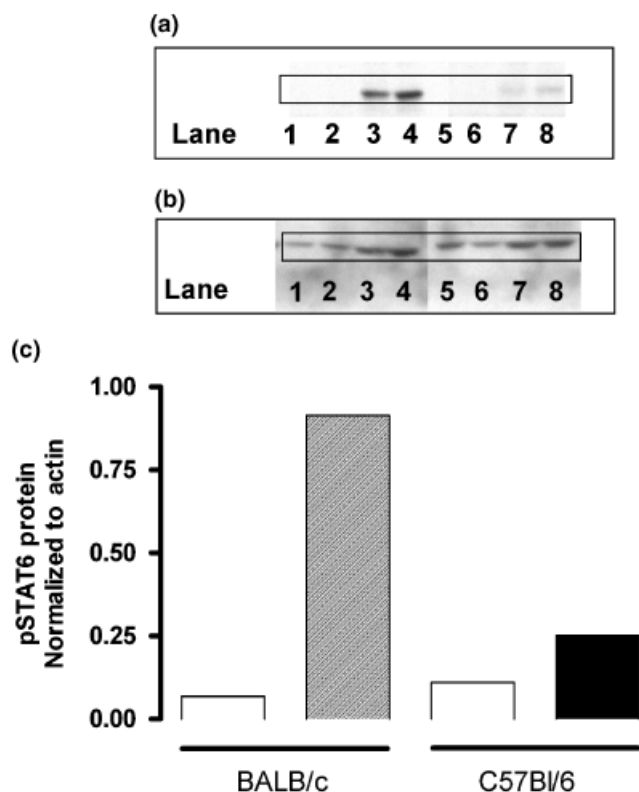


Fig. 4. Immunoblot for pSTAT6 in brief allergen-exposed (24-h recovery) BALB/c and C57Bl/6 mice: (a) pSTAT6; (b) actin; (c) mean normalization ($n = 2$) of pSTAT6 signal to actin level using the densitometry application in BioRad Quantity One Software. Lane 1: BALB/c brief SAL; Lane 2: BALB/c brief SAL; Lane 3: BALB/c brief ovalbumin (OVA); Lane 4: BALB/c brief OVA; Lane 5: C57Bl/6 brief SAL; Lane 6: C57Bl/6 brief SAL; Lane 7: C57Bl/6 brief OVA; Lane 8: C57Bl/6 brief OVA. BALB/c saline: open white bar; BALB/c OVA: hatched white bar; C57Bl/6 saline: open white bar; C57Bl/6 OVA: solid black bar.

Airway physiology following brief allergen exposure

Following a single 2-day period of allergen exposure, airway physiology measurements were performed in BALB/c and C57Bl/6 mice (Fig. 5). In contrast to the observation of similar inflammatory responses in both strains, but consistent with the STAT6 signalling, significant increases in airway reactivity to methacholine and maximum induced airway resistance were observed in BALB/c, but not C57Bl/6 mice ($P < 0.05$; Fig. 5).

Activity of STAT6 and SMAD2 pathways following chronic allergen exposure

STAT6 phosphorylation was maintained throughout chronic allergen exposure in BALB/c mice (Fig. 6a). Significant increases in positive-stained cells were observed in BALB/c mice with the nuclear staining localized to small, nuclear dense, inflammatory cells with lymphocyte morphology ($P < 0.05$; Fig. 6c). In contrast to earlier

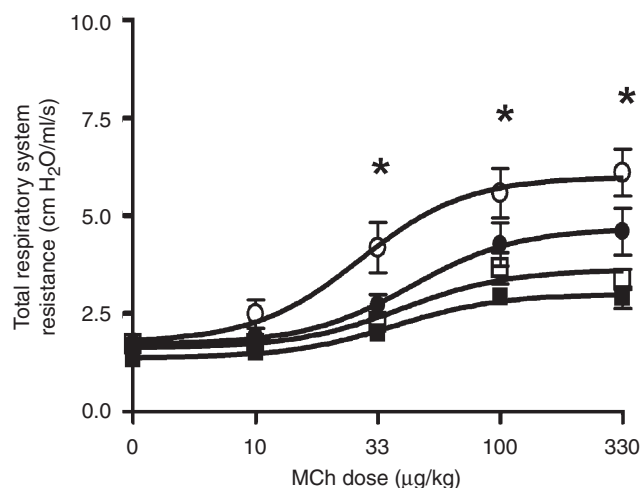


Fig. 5. Airway physiology in mice following brief allergen exposure. Dose response curve to saline and increasing doses of intravenous methacholine. BALB/c saline: solid black circle; BALB/c ovalbumin (OVA): open white circle; C57Bl/6 saline: solid black square; C57Bl/6 OVA: open black square. Data are expressed as mean (SEM); 10 mice per group. * indicates significant difference from corresponding control animals ($P < 0.05$).

time-points, significant increases in pSTAT6 staining were observed in C57Bl/6 mice following chronic allergen exposure ($P < 0.05$; Fig. 6a). Interestingly, pSTAT6 staining was restricted to large, single-nucleated, inflammatory cells with macrophage morphology and was completely cell membrane localized (Fig. 6e).

The delayed increase in pSMAD2 elevation in BALB/c mice was still detectable 2 weeks post cessation of chronic allergen exposure ($P < 0.05$; Fig. 6f). No elevations in pSMAD2 levels were detected in C57Bl/6 mice following chronic allergen exposure (Fig. 6f).

To confirm and support our immunohistochemistry of pSMAD2 staining, we performed immunoblot analysis on BALB/c and C57Bl/6 mice 2 weeks following chronic allergen exposure. Consistent with our immunohistochemistry, greater elevations in pSMAD2 levels were detected in BALB/c than C57Bl/6 mice (Fig. 7a–c).

Structural airway changes following chronic allergen exposure

Despite similar inflammatory events in BALB/c and C57Bl/6 mice, significant changes in airway structure were restricted to the BALB/c mice, consistent with the early changes in STAT6 and SMAD2 signalling in these mice.

Four weeks following chronic allergen exposure, at a time-point when ongoing inflammation had resolved [13], there were significant increases in airway goblet cell number in BALB/c mice, but not C57Bl/6, compared with control animals ($P < 0.05$; Fig. 8a).

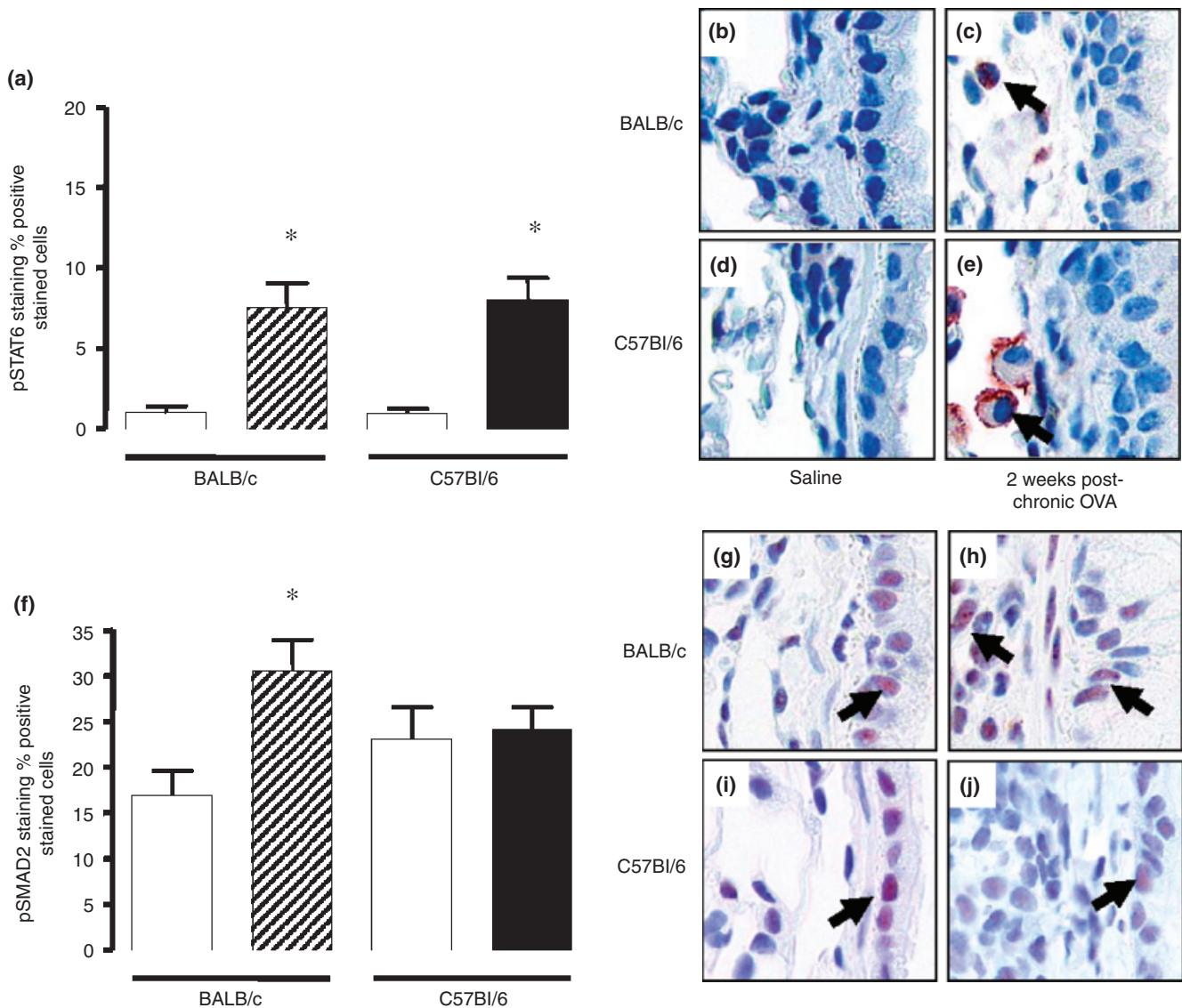


Fig. 6. STAT6 and SMAD2 phosphorylation following chronic allergen exposure. (a) Morphometric analysis of pSTAT6 staining in mice following brief allergen exposure (positive-stained cells/total cell number $\times$ 100). (b–e) Representative samples of pSTAT6 staining following chronic allergen exposure: (b) BALB/c chronic saline exposed; (c) BALB/c chronic OVA exposed – 2-week recovery period; (d) C57Bl/6 chronic saline exposed; (e) C57Bl/6 chronic OVA exposed – 2-week recovery; (f) morphometric analysis of pSMAD2 staining in mice following chronic allergen exposure (positive-stained cells/total cell number $\times$ 100). (g–j) Representative samples of pSMAD2 staining following chronic allergen exposure: (g) BALB/c chronic saline exposed; (h) BALB/c chronic OVA exposed – 2-week recovery period; (i) C57Bl/6 chronic saline exposed; (j) C57Bl/6 chronic OVA exposed – 2-week recovery. BALB/c saline: open white bar; BALB/c OVA: hatched white bar; C57Bl/6 saline: open white bar; C57Bl/6 OVA: solid black bar. Data are expressed as mean (SEM); 10 mice per group. * indicates significant difference from corresponding control animals ($P < 0.05$).

Consistent with the changes in goblet cells, there were significant increases in subepithelial collagen content in BALB/c mice, but not C57Bl/6, compared with control animals ($P < 0.05$; Fig. 8b) as assessed by quantitative airway morphometry [14].

Finally, and again consistent with other structural changes, there were significant increases in the smooth muscle content in BALB/c mice, but not C57Bl/6, compared with control animals ($P < 0.05$; Fig. 8c) as assessed by quantitative airway morphometry [14].

Airway physiology following chronic allergen exposure

Airway function measurements were made 4 weeks following chronic allergen exposure (Fig. 8d), at a time-point when ongoing inflammation had resolved [13]. At this time, consistent with the differences in indices of airway remodelling, significant increases in airway responsiveness was observed in BALB/c, but not C57Bl/6 mice, as compared with control animals ($P < 0.05$; Fig. 8d).

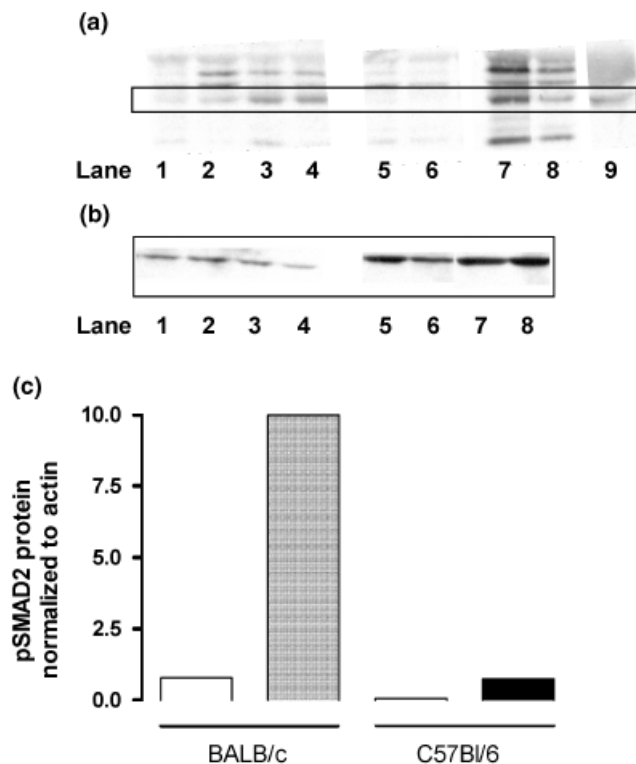


Fig. 7. Immunoblot for pSMAD2 in chronic allergen-exposed (2-week recovery) BALB/c and C57Bl/6 mice: (a) pSMAD2; (b) actin; (c) normalization of pSMAD2 signal to actin level using the densitometry application in BioRad Quantity One Software. Lane 1: BALB/c chronic SAL; Lane 2: BALB/c chronic SAL; Lane 3: BALB/c chronic ovalbumin (OVA); Lane 4: BALB/c chronic OVA; Lane 5: C57Bl/6 chronic OVA; Lane 6: C57Bl/6 chronic OVA; Lane 7: C57Bl/6 chronic SAL; Lane 8: C57Bl/6 chronic SAL; Lane 9: TGF- β 1-treated rat peritoneal dialysis fluid (positive control), BALB/c saline: open white bar; BALB/c OVA: hatched white bar; C57Bl/6 saline: open white bar; C57Bl/6 OVA: solid black bar.

Role of IL-4 and IL-13 in STAT6 and SMAD2 phosphorylation

BALB/c mice clearly demonstrate phosphorylation of STAT6, and this appears to be associated with the nucleus. Because STAT6 phosphorylation can be the result of either IL-4 or IL-13 activation, we sought to determine the independent contribution of these cytokines in BALB/c mice using genetically deficient animals at a time-point when pSTAT6 staining was elevated in wild-type animals.

Following brief allergen exposure, OVA-exposed BALB/c IL-4^{-/-} mice demonstrated an increase in pSTAT6 staining similar to that shown by wild-type OVA-exposed mice ($P < 0.05$; Fig. 9a–d). Conversely, OVA-exposed BALB/c IL-13^{-/-} mice demonstrated significantly reduced pSTAT6 staining as compared with wild-type OVA-exposed mice ($P < 0.05$; Fig. 9a–d). The staining in OVA-exposed BALB/c IL-13^{-/-} mice was similar to that in saline-exposed wild-type mice (Figs 9d and 3b).

Activation of the SMAD2 pathway can be induced by IL-13 [5, 11]. As such, we set out to determine the

contribution of IL-4 and IL-13 in the phosphorylation of SMAD2.

Following chronic allergen exposure, OVA-exposed BALB/c IL-4^{-/-} mice maintained detectable levels of pSMAD2 similar to those maintained by wild-type OVA mice (Fig. 9e–h). Conversely, OVA-exposed BALB/c IL-13^{-/-} demonstrated significantly reduced pSMAD2 staining as compared with wild-type OVA animals ($P < 0.05$; Fig. 9e–h). The staining in OVA-exposed BALB/c IL-13^{-/-} mice was similar to that in saline-exposed wild-type mice (Figs 9h and 6g).

Discussion

The mechanisms responsible for airway remodelling and the relative contribution of these structural changes to AHR are poorly understood. Using two genetically distinct strains of mice, we demonstrate that activation of signalling mechanisms downstream from inflammatory mediators is more closely associated with the development of airway remodelling and airway dysfunction, as compared with levels of cells and mediators themselves. Specifically, ongoing Th2 inflammation was observed in both BALB/c and C57Bl/6 mice, although only BALB/c mice progressed to develop structural changes and sustained functional changes. We demonstrate that following brief allergen exposure, STAT6 phosphorylation precedes that of SMAD2, that both these signals are sustained throughout extended periods of chronic allergen exposure, are associated with airway remodelling, and are IL-13 dependent. Finally, we demonstrate that reduced and distinct patterns of STAT6 phosphorylation are observed in C57Bl/6 mice. By observing similar inflammatory profiles in BALB/c and C57Bl/6 mice, despite disparate development of functional and pathological changes, we suggest that the presence of airway inflammation alone may not be sufficient for chronic disease progression in wild-type mice. Rather, our data suggests that activation of signalling pathways downstream from elevations in inflammatory mediators may be more relevant in disease progression and should be targeted for development of novel therapeutics (e.g. STAT6/SMAD2 nuclear localization inhibitors).

Attempts to delineate mechanisms involved in the development of airway remodelling commonly employ genetically modified animals. Results of studies with gene-deficient animals should be carefully interpreted, as compensatory/developmental problems can never be entirely ruled out [19]. In this context, several experiments have investigated the role of STAT6 in murine models of allergen exposure using STAT6^{-/-} animals [20, 21]. Seminal work by Kuperman *et al.* [21] clearly demonstrated that in a brief allergen model with a single exposure period, BALB/c STAT6^{-/-} mice were protected from development of allergen-induced increased airway responsiveness. Further, STAT6^{-/-} mice were resistant to

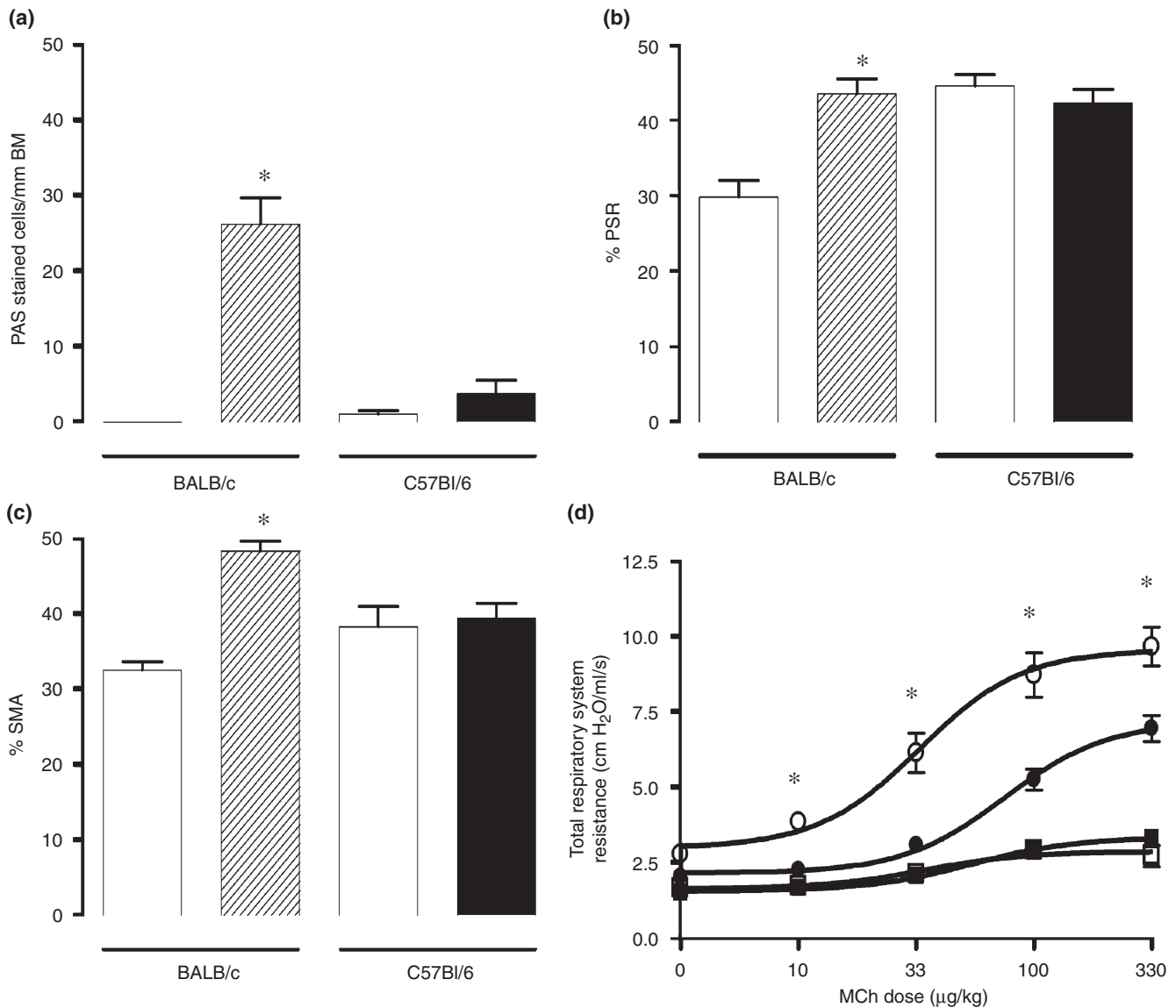


Fig. 8. Morphometric analysis of airways and airway physiology responses following chronic allergen exposure. (a) Periodic acid-Schiff (PAS) staining for goblet cells (number of cells/mm basement membrane). (b) Picrosirius red (PSR) staining for collagen deposition (% stain in region of interest). (c) α -Smooth muscle actin (α -SMA) staining for smooth muscle content (% stain in region of interest). BALB/c saline: open white bar; BALB/c ovalbumin (OVA): hatched white bar; C57Bl/6 saline: open white bar; C57Bl/6 OVA: solid black bar. Data are expressed as mean (SEM); 10 mice per group. Asterisk (*) indicates significant difference from corresponding control animals ($P < 0.05$). (d) Airway physiology in mice following chronic allergen exposure. Dose response curve to saline and increasing doses of intravenous methacholine. BALB/c saline: solid black circle; BALB/c OVA: open white circle; C57Bl/6 saline: solid black square; C57Bl/6 OVA: open black square.

goblet cell metaplasia and mucus production in this single exposure model, although airway eosinophilia was still detected. Interestingly, long-term allergen exposure protocols in STAT6^{-/-} mice may lead to discrepant results; it has been shown that following chronic allergen exposure, BALB/c STAT6^{-/-} mice are indeed capable of developing eosinophilia, epithelial changes, thickening of subepithelium and increases in AHR [20]. These seemingly disparate findings must be considered in determining the role of STAT6 in an experimental model, and lead us to incorpo-

rate various time-points in brief and chronic allergen exposure protocols. Using this approach, we demonstrate that early activation of the STAT6 pathway in BALB/c mice is not only sustained following allergen exposure, but also associated with the development of acute inflammatory and chronic remodelling events. Our findings are consistent with the hypothesis that IL-13 is required for the development of transient AHR following brief exposure to allergen [2], and several indices of remodelling, including goblet cell hyperplasia, increase in smooth

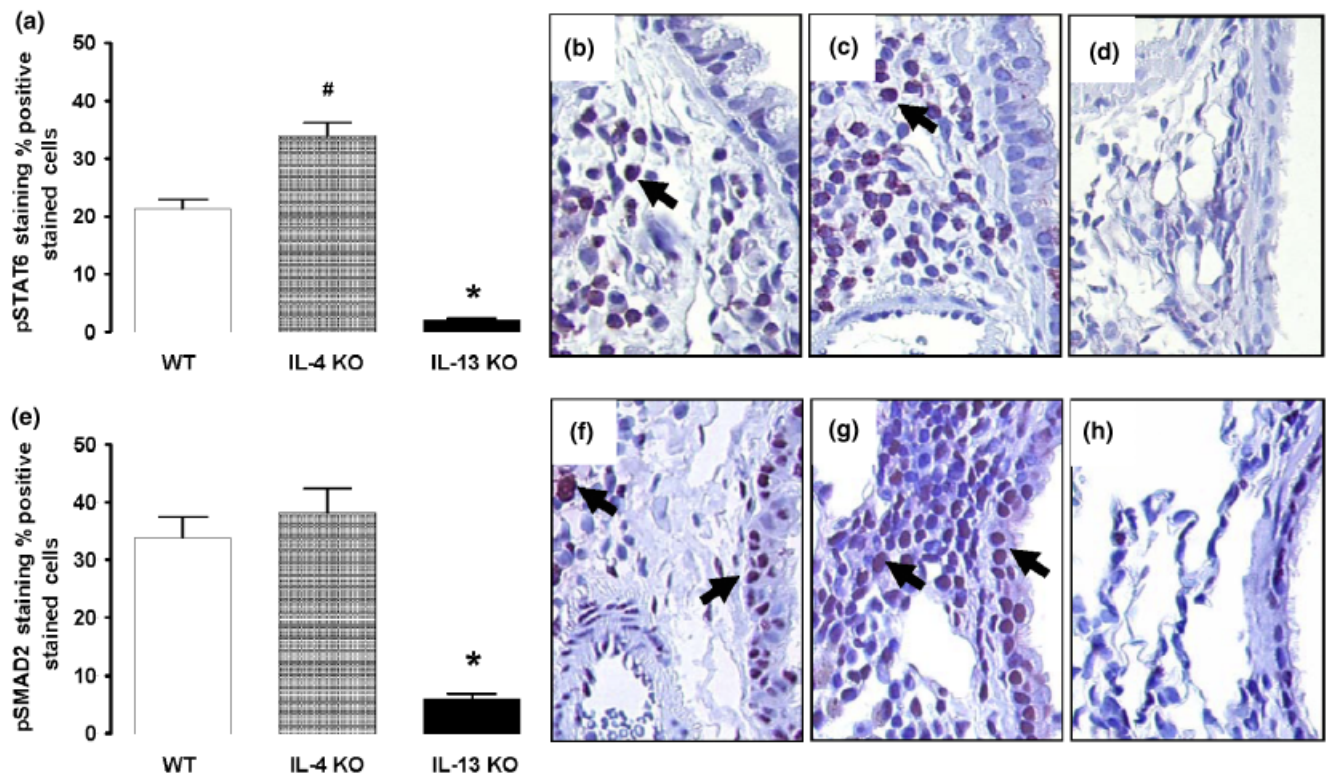


Fig. 9. STAT6 and SMAD2 phosphorylation in IL-4 and IL-13-deficient mice. (a) Morphometric analysis of pSTAT6 staining in mice following brief allergen exposure (positive-stained cells/total cell number $\times 100$). (b–d) Representative examples of pSTAT6 staining in mice following brief allergen exposure ($n = 5$). Arrows indicate positively stained cells: (b) BALB/c wild-type mouse; (c) BALB/c IL-4^{-/-} mouse; (d) BALB/c IL-13^{-/-} mouse; (e) morphometric analysis of pSMAD2 staining in mice following chronic allergen exposure (positive-stained cells/total cell number $\times 100$). (f–h) Representative samples of pSMAD2 staining following chronic allergen exposure ($n = 5$). Arrows indicate positively stained cells: (f) BALB/c wild-type mouse; (g) BALB/c IL-4^{-/-} mouse; (h) BALB/c IL-13^{-/-} mouse. BALB/c saline: open white bar; BALB/c ovalbumin (OVA): hatched white bar; C57Bl/6 saline: open white bar; C57Bl/6 OVA: solid black bar. Data are expressed as mean (SEM); 10 mice per group. Asterisk (*) indicates significantly lower levels as compared to wild-type (WT) animals ($P < 0.05$). Number symbol (#) indicates significantly higher levels as compared to WT animals ($P < 0.05$).

muscle and sub-epithelial fibrosis, following chronic allergen exposure [3]. Additionally, we present data that strongly suggests that a marked increase in STAT6 phosphorylation during allergen exposure in BALB/c mice is dependent on IL-13, but not IL-4. Interestingly, when IL-4 is knocked out, we see greater STAT6 phosphorylation, which although completely conjecture may be the result of different binding thermodynamics between IL-4 and IL-13 and their respective receptors and their coupling efficiency to STAT6 [22].

Our strain-dependent results could be in part explained by previously described polymorphisms in the IL-4 α receptor [23]. Webb *et al.* demonstrated that the IL-4 α chains in BALB/c and C57Bl/6 mice are different by nine amino acids. Interestingly, mice carrying the C57Bl/6 sequence of the IL-4 α chain developed AHR, eosinophilia and elevations in IgE levels that were IL-4 dependent, while mice containing the BALB/c sequence of the IL-4 α chain were resistant to these changes. Although these data may be somewhat contradictory to our findings, they suggest that additional sequence differences might exist in the signalling pathways of these two strains (e.g. STAT6

or nuclear import factors) that may lead to variations in post-translational modifications [23].

Of particular interest is the initial distribution of IN-instilled solutions in the lungs of BALB/c and C57Bl/6 mouse strains. It is possible that time-dependent distribution patterns in the lower and upper airways may differ between strains leading to differential development of immune responses associated with lymphatic tissues in these areas. A time course study imaging the distribution of longer lasting radiolabelled compounds (18-FDG half-life = 2 h) in BALB/c and C57Bl/6 mice may provide insight into early immune responses.

The TGF- β 1 pathway is known to be implicated in a variety of fibrotic diseases, and its activity is often demonstrated by transcript expression or total/active protein levels. Recently, it has been suggested that a more suitable marker for TGF- β 1 activity is the phosphorylation of its downstream effector, SMAD2 [17, 18]. Consistent with this, we detected TGF- β 1 mRNA in the tissue, but not active protein in the lavage fluid from airways of BALB/c mice following brief allergen exposure (data not shown). Our results suggest that TGF- β 1 activity is not immediately

detectable following allergen exposure but instead ramps up during recovery or continued allergen exposure. It is tempting to suggest that our data supports the inflammation–damage–repair–remodelling paradigm considered to be responsible for airway remodelling [24], although our data remains correlative at this point. Our data support a link between the STAT6 and SMAD2 pathways, as SMAD2 phosphorylation was dependent on IL-13 in BALB/c mice following allergen exposure, in addition to positive associations between TGF- β 1 gene expression and pSTAT6 levels. Although the temporality of our data is consistent with the inflammation/damage/repair/remodelling hypothesis, it is clear that further experiments with specific interventions (e.g. STAT6 inhibitors) are required to test this hypothesis and provide complementary mechanistic data.

The observations of allergic sensitization, Th2-skewed inflammation and airway eosinophilia in C57Bl/6 mice that are not followed by structural and functional changes are intriguing. Interestingly, we did not see activation of the STAT6 pathway following brief allergen exposure. Even when the STAT6 pathway was activated following chronic allergen exposure, the signal appeared to be membrane localized and detectable only in a cell morphologically distinct from that observed in BALB/c mice. It is possible that despite C57Bl/6 mice developing Th2 inflammatory markers, associated downstream events, including goblet cell metaplasia, increased levels of TGF- β 1, pSMAD2 and fibrosis, require nuclear localization of pSTAT6 in a specific cell type. The distinct patterns of pSTAT6 staining in BALB/c and C57Bl/6 mice suggest that intracellular signalling, secondary to increased levels of IL-4/IL-13, differ between these strains. To date, no reports of functional consequences of polymorphisms in STAT6 have been reported for different mouse strains or humans. Additionally, the presence of nuclear localization signals within STAT6 and/or the mechanisms responsible for nuclear translocation remain to be determined [25]. Clearly, our findings suggest that a better understanding of the mechanisms responsible for interfering with this signalling pathway may uncover novel therapeutic targets.

The contribution of airway inflammation to AHR can be explored in short-term allergen exposure models, while to accurately examine the role of airway remodelling, we must use chronic exposure models – in the absence of ongoing inflammation. Using both brief and chronic allergen exposure models, we demonstrate that prolific Th2-skewed inflammation is associated with AHR in BALB/c mice. Th2 inflammation is accompanied by early activation of the STAT6 pathway, delayed activity of the SMAD2 pathway and development of indices of airway remodelling and sustained AHR in BALB/c mice. Interestingly, in our model, C57Bl/6 mice are resistant to the development of airway remodelling and sustained AHR despite prolific Th2 inflammation, perhaps the consequence of an abnormal STAT6 signalling pathway.

Mechanisms responsible for pSTAT6 nuclear translocation may provide a suitable therapeutic target for the treatment of airway inflammation and remodelling.

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Supporting information

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

Fig. S1. Inflammatory profiles of mice following brief allergen exposure. (a) Serum IgE levels determined by ELISA (level of detection 1.6 ng/mL). (b) Bronchoalveolar lavage (BAL) supernatant IL-13 levels (level of detection 19.5 pg/mL). (c) BAL supernatant IL-4 levels (level of detection 7.8 pg/mL). (d) BAL supernatant IFN- γ levels (level of detection 3.1 pg/mL). Individual data points are presented, as a large majority of samples were below manufacturer recommended level of detection, and a bar graph could not be generated. (e) BAL eosinophil counts ($\times 10^4$ cells). (f) Tissue eosinophil counts (eosinophils/ μm^2). BALB/c saline: open white bar; BALB/c ovalbumin (OVA): hatched white bar; C57Bl/6 saline: open white bar; C57Bl/6 OVA: solid black bar. Data are expressed as mean (SEM); 10 mice per group. Dashed lines represent the level of detection of ELISA kit. Asterisk (*) indicates significant difference from corresponding control animals ($P < 0.05$).

Fig. S2. TGF- $\beta 1$ gene expression in lungs from BALB/c and C57Bl/6 mice following brief allergen exposure. Gene expression was performed 2 weeks following a single 2-day period of allergen exposure (Day 34) in BALB/c mice and C57Bl/6 mice. BALB/c saline: open white bar; BALB/c ovalbumin (OVA): hatched white bar; C57Bl/6 saline: open white bar; C57Bl/6 OVA: solid black bar. Data are expressed as mean (SEM); eight mice per group.

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Online Supplement

The role of STAT6 and SMAD2 in a model of chronic allergen exposure : A mouse strain comparison study

Jeremy A. Hirota, Kjetil Ask, Dominik Fritz, Russ Ellis, Jennifer Wattie, Carl D. Richards, Renee Labiris, Martin Kolb, Mark D. Inman

Methods

Immunoblots: Immunoblot primary antibodies (Abs) specific for phosphotyrosine pY641 STAT6 (pSTAT6) and phosphoserine pS465/467 SMAD2 (pSMAD2) were purchased from Cell Signaling Technology Incorporated (Danvers, Mass). Primary Abs specific for actin (I-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Primary Abs against pSTAT6 were diluted 1/1000 in 5.0% BSA (Sigma-Aldrich, Oakville, Ontario). Primary Abs against STAT6, pSMAD2, and actin were diluted 1/1000 in 5.0% milk. For detection of primary pSTAT6 and STAT6, secondary goat anti-mouse IgG HRP Ab was purchased from Santa Cruz Biotechnology. Goat anti-mouse IgG HRP Ab was diluted 1/2500 in 5.0% milk. For detection of primary pSMAD2, secondary goat anti-rabbit IgG HRP Ab was purchased from Santa Cruz Biotechnology. Goat anti-rabbit IgG HRP Ab was diluted 1/2500 in 5.0% milk. For 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 radioimmunoprecipitation (RIPA) lysis buffer (1x PBS (pH 7.4), 1.0% igeal CA-630, 0.5% sodium deoxycholate ($C_{24}H_{39}NaO_4$), 0.1% SDS, including inhibitors (1 mM PMSF, 1 mM sodium orthovanadate (Na_3VO_4), and 1 mg/ml aprotinin). Lung homogenates were incubated for 1 hour on ice.

Lung homogenates were then vigorously sheared using a syringe with a 21-gauge needle, centrifuged at maximum speed (12,000 x 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). Equal amounts of protein were loaded onto 8% SDS-PAGE gels for electrophoresis. Proteins were transferred to Immobilon-NC membranes (Millipore, Ottawa, Ontario). Immunoblots were blocked in 1x 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 hour at room temperature, and visualized using an ECL Western Blotting Analysis System (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec).

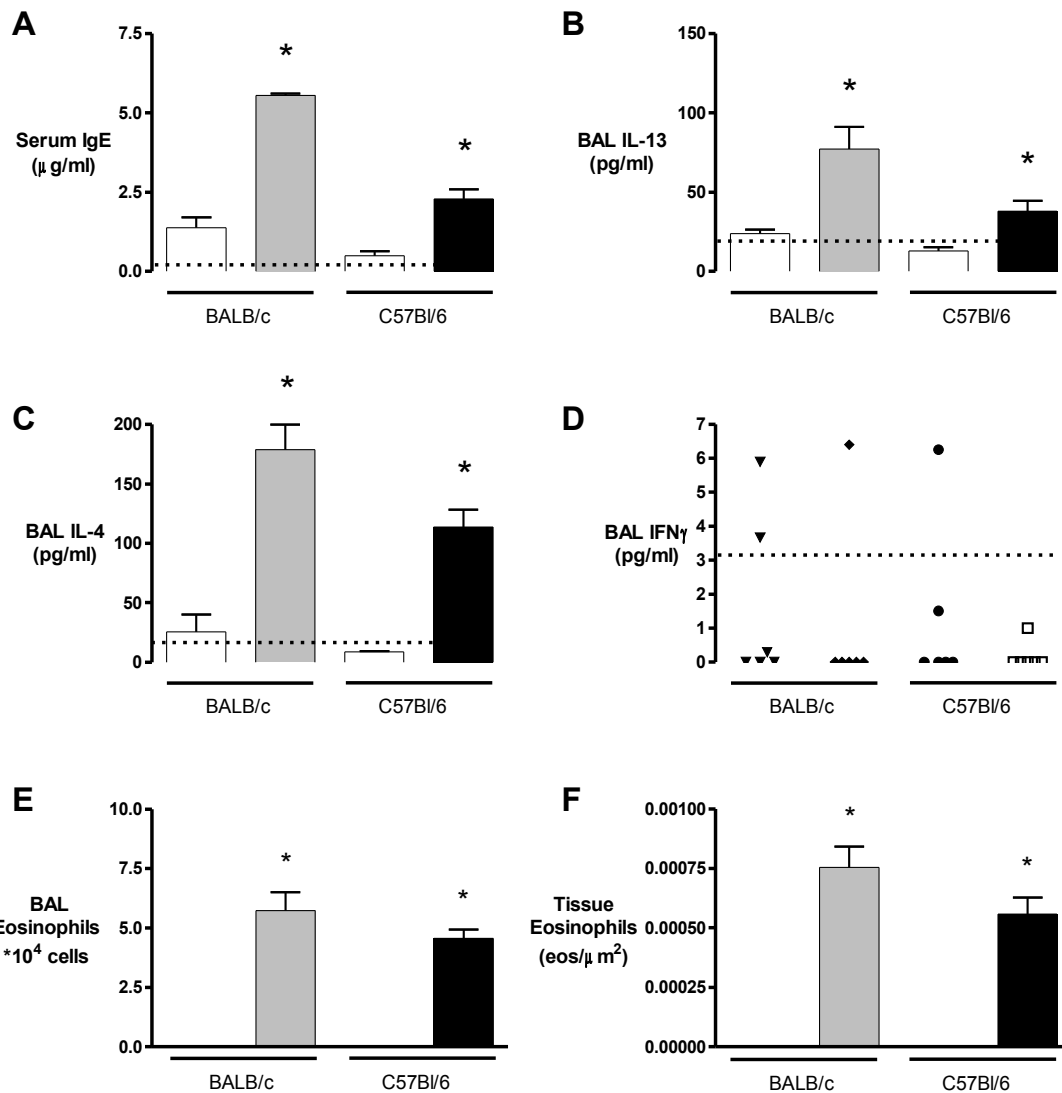
Immunoblots were exposed by X-OMAT Kodak Scientific Films and images scanned at 300–400 dpi resolution. Band density was analyzed for pSTAT6, STAT6, pSMAD2 and loading control (actin) using BioRad Quantity One software. Density levels for pSTAT6, STAT6, and pSMAD2 were normalized using density values of the loading control (actin) and fold induction for these proteins was quantified.

Gene Expression Analysis : Gene expression analysis was performed 2wks following a single period of allergen exposure (Day 34). Following euthanasia, lungs were removed, rinsed in PBS, and immediately immersed in RNAlater (Ambion, Streetsville, ON, Canada) and stored at 4°C elsius overnight as per the manufacturer's recommendations. RNA isolation was performed using a Qiagen MiniPrepKit (Mississauga, ON, Canada) and concentration and purity were assessed by optical density measurements. RNA was transcribed using a standard protocol (Invitrogen Life Technologies, Burlington, ON). Quantitative real-time PCR was conducted using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, U.S.A.). Negative control samples (no template or no

reverse transcriptase) were run concurrently. Relative quantification was performed (ddCT method) with results normalized to GAPDH and all values expressed as a fold increase over a naïve C57Bl/6 whole lung homogenate. Assay on Demand products for TGF- β 1 and GAPDH were purchased from Applied Biosystems.

Lung Deposition PET/CT study – Mouse Strain Comparison: Animals were anaesthetized using isoflurane. Approximately 200 uCi of 18-FDG in 25 μ l of PBS was administered intranasally (IN) using the same technique used to deliver the allergen. Immediately after IN delivery, a whole body static scan using a small animal positron emission tomography (PET, Mosaic PET, Philips Healthcare) was acquired followed by a computed tomography (CT). The PET and CT images were fused to determine the anatomical location of the radioactivity. Radioactivity counts were adjusted for amount of activity administered and body weight of the animal. Regions of interest were drawn over the lung, nose, and whole body and radioactivity in the areas were determined.

Results:



Supplement Figure 1 : Inflammatory profiles of mice following brief allergen exposure. **A** – Serum IgE levels determined by ELISA (level of detection 1.6 ng/ml). **B** – BAL supernatant IL-13 levels (level of detection 19.5 pg/ml). **C** – BAL supernatant IL-4 levels (level of detection 7.8 pg/ml). **D** – BAL supernatant IFN- γ levels (level of detection 3.1 pg/ml). Individual data points are presented as the large majority of samples were below manufacturer recommended level of detection and a bar graph could not be generated. **E** – BAL eosinophil counts ($\times 10^4$ cells). **F** – Tissue eosinophil counts (eosinophils/ μm^2).

BALB/c Saline – open white bar

BALB/c OVA – hatched white bar

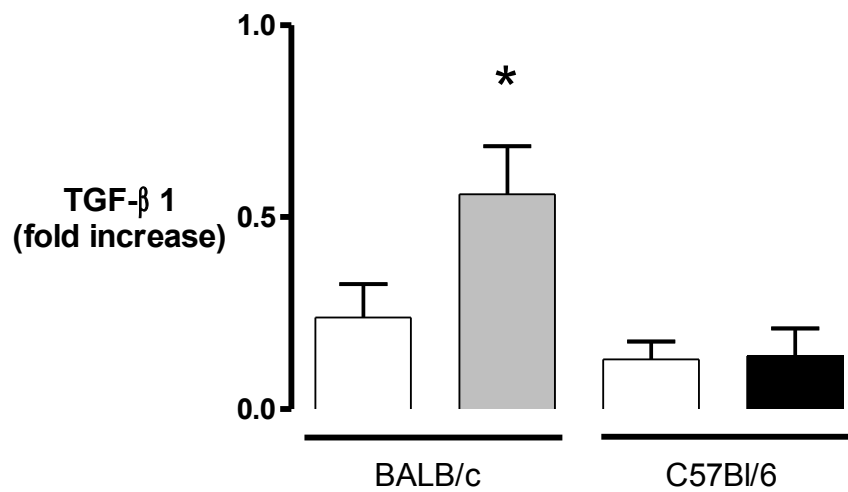
C57Bl/6 Saline – open white bar

C57Bl/6 OVA – solid black bar

Data are expressed as mean (SEM); 10 mice per group.

Dashed lines represent level of detection of ELISA kit

* significantly different from corresponding control animals ($p < 0.05$)



Supplement Figure 2 : TGF- β 1 gene expression in lungs from BALB/c and C57Bl/6 mice following brief allergen exposure. Gene expression was performed 2wks following a single 2-day period of allergen exposure (Day 34) in BALB/c mice and C57Bl/6 mice.
BALB/c Saline – open white bar
BALB/c OVA – hatched white bar
C57Bl/6 Saline – open white bar
C57Bl/6 OVA – solid black bar
Data are expressed as mean (SEM); 8 mice per group.