British Journal of Pharmacology (2000) 130, 1581–1588 © 2000 Macmillan Publishers Ltd All rights reserved 0007–1188/00 $15.00
www.nature.com/bjp

Abrogation of lung inflammation in sensitized Stat6-deficient mice is dependent on the allergen inhalation procedure

*,[1]Alexandre Trifilieff, [1]Ahmed El-Hasim, [1]Randolph Corteling & [1]Charles E. Owen

[1]Novartis Horsham Research Centre, Wimblehurst Road, Horsham, RH12 5AB

1 Conflicting results have been reported about the role of Stat6 in allergen-induced airway inflammation. We have studied the influence of the allergen inhalation procedure on the inflammatory response using wild-type and Stat6-deficient mice generated on a C57BL/6 background.

2 Animals were immunized i.p. on day 0 and 7 with ovalbumin (OVA) and then received aerosolized OVA or phosphate buffer saline challenge (acute on day 14; chronic on day 14, 15, 16, 17 and 18) before being sacrificed at different time points.

3 Following an acute challenge, Stat6-deficiency fully abrogated the increase in serum IgE levels and the development of lung inflammation (inflammatory cell infiltration, IL-4 and IL-5 release, and increase in plasma leakage).

4 Following chronic challenge, despite the absence of IgE, IL-4 and IL-5, Stat6-deficient mice develop a characteristic lung inflammation, although the intensity was smaller when compared with the wild-type mice.

5 OVA-induced early bronchoconstriction was observed in wild-type mice only after chronic challenge, and this was totally abrogated in the Stat6-deficient animals.

6 These results suggest that Stat6 signalling is essential for the development of allergic airway inflammation following an acute allergen exposure. However, in a more chronic situation, the airway inflammatory response seems to be only partially mediated by Stat6.
British Journal of Pharmacology (2000) **130,** 1581–1588

Keywords: Rodent; lung; inflammation; transcription factors; *in vivo* animal models

Abbreviations: BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase; i.p., intraperitoneal.; NF-κB, nuclear factor kappa B; OVA, ovalbumin; PBS, phosphate buffer saline; Stat, signal transducer and activator of transcription; TH2, T helper 2; VCAM-1, vascular adhesion molecule-1

Introduction

Bronchial asthma is characterized by episodes of bronchoconstriction, increased mucous secretion and airway infiltration with eosinophils and mononuclear cells (Djukanovic *et al*., 1990). Allergen-induced asthma is an important form of asthma and there is good evidence to link this form of the disease with a high titre of circulating IgE (Oettgen & Geha, 1999). Following allergen exposure, patients with allergen-induced asthma develop an early bronchoconstriction response. This response is dependent, at least in part, on IgE-mediated mast cell degranulation (Casale *et al*., 1987). In many patients, this early-response is followed by a late-response occurring 8 to 24 h after the allergen exposure and characterized by an airway infiltration with eosinophils and mononuclear cells (Djukanovic *et al*., 1990).

Clinical and experimental investigation have suggested that allergen-specific CD4+T helper 2 (Th2)-type cells and Th2 cytokines play a central role in initiating and sustaining an asthmatic response by regulating the recruitment and/or activation of airway inflammatory cells (Walker *et al*., 1991; Robinson *et al*., 1992). Among these, IL-4 is critical in the commitment of CD4+T-cells to the Th2 phenotype and for IgE isotype switching in B-cells (Bergstedt-Lindqvist *et al*., 1988; Swain *et al*., 1990). Murine models of asthma using IL-4 deficient mice or the exogenous application of cytokines or cytokine antibodies, has resulted in conflicting data (Brusselle *et al*., 1995; Coyle *et al*., 1995; Hogan *et al*., 1997). IgE, produced by B-cells when stimulated with IL-4 is believed to be an important mediator in allergic asthma. However, there is conflicting evidence about the importance of IgE in the molecular mechanisms underlying the allergen-induced airway inflammation in mice (Coyle *et al*., 1996; Mehlhop *et al*., 1997; Korsgren *et al*., 1997).

Recently, it has been demonstrated that members of the signal transducer and activator of transcription (Stat) gene family are critical in the intracellular pathway of many cytokine receptors (Ihle, 1995). Of these, Stat6 is involved in the vast majority of IL-4-induced responses including Th2 differentiation and Ig class switching to IgE (Kaplan *et al*., 1996; Shimoda *et al*., 1996) which are key events in the asthma allergic response. Previous studies, using murine models of asthma and Stat6-deficient mice have shown different results. In two of these studies the bronchial inflammation and airway hyperreactivity were totally abrogated in the deficient mice (Akimoto *et al*., 1998; Tomkinson *et al*., 1999), whereas in another study although the airway hyperreactivity was fully abolished, the airway inflammation was only partially inhibited in the deficient mice (Kuperman *et al*., 1998). In all these studies there was a lack of consistency in the mice strains and the allergen provocation protocols. Because the influence of the protocol of allergen provocation and the genetic background of the animals has been shown to be important in the murine allergic airway response (Blyth *et al*., 1996; Zhang *et al*., 1997) consequently, we re-examined the role

*Author for correspondence.

played by Stat6 following acute or chronic allergen challenges on actively sensitized Stat6-deficient mice generated on a C57/BL6 background. To this end, we compared the effect of two challenge protocols (acute and chronic) on the development of allergen-induced early bronchoconstriction; total serum IgE production; airway inflammatory cells influx; BAL cytokines, eosinophil peroxidase (EPO) activity and fibronectin levels in wild-type and Stat6-deficient mice.

Methods

Animals

C57/BL6 mice with targeted disruption of the gene encoding Stat6 were provided by Dr Jim Ihle (St Jude Children Research Hospital, Memphis, TN, U.S.A.) and inbred by B&K universal (North Humberside, U.K.). Age and sex matched C57/BL6 mice were purchased from Harlan (Oxon, U.K.). The animals were housed in plastic cages in an air-conditioned room at 24°C. Food and water were available *ad libitum*. The studies reported here conformed to the U.K. Animal (scientific procedures) Act 1986.

Immunization and exposure of mice

Animals were immunized intraperitoneally (i.p.) with 20 μg of OVA, (grade V, Sigma, St. Louis, MO, U.S.A.) in 0.2 ml of alum (Serva, Heidelberg, Germany) on day 0 and day 7. Sham-immunized mice received two injections of alum alone. From day 14 animals were exposed, for 20 min, to an aerosol of OVA in phosphate buffer saline (PBS) (50 mg ml^{-1}) or PBS alone using a nose only exposure system. The effects of OVA challenge were studied after a single challenge (acute model) or after a challenge on each of 5 consecutive days (chronic model). At the specified time point after the last challenge, terminal anaesthesia was induced with pentobarbitone sodium (60 mg kg^{-1}, i.p.).

Assessment of BAL inflammatory cell infiltration

After anaesthesia, a blood sample was taken from the abdominal aorta for serum IgE level determination, then the animals were exsanguinated by withdrawal of blood from a major blood vessel. The trachea was cannulated and bronchoalveolar lavage (BAL) was performed by injecting four times 0.3 ml of PBS into the lung *via* the trachea. The fluid was immediately withdrawn and the cell suspension stored on ice. Total cell count was measured and cytospin preparation (Shandon Scientific Ltd, Cheshire, U.K.) prepared. Cells were stained with Dif-Quik (Baxter Dade AG, Dudingen, Switzerland) and a differential count of 200 cells performed using standard morphological criteria. The remaining lavage fluid was centrifuged at $200 \times g$ for 10 min, the supernatant was aliquoted and stored at -80°C.

BAL soluble mediators measurement

BAL cytokines levels (IL-5, IL-4, IFN-γ) were measured using commercially available kits (Endogen, Wolburn, MA, U.S.A.). The sensitivity of these assays was 15 pg ml^{-1} for INF-γ and IL-5 and 5 pg ml^{-1} for IL-4.

The quantification of eosinophil peroxidase (EPO) activity was performed in 96-well plates, by a cytochemical enzyme assay on fresh BAL supernatant. A standard curve was established using a serial dilution of commercial horseradish peroxidase (Sigma, St Louis, MO, U.S.A.) starting at 1 u ml^{-1}. Fifty μl of the samples were incubated for 30 min with 100 μl of *o*-phenylenediamine dihydrochloride (1 mM) in Tris-HCl buffer (0.05 M, pH 8) containing 0.1% Triton X-100 and 0.0005% H_2O_2. The reaction was stopped by adding 50 μl of 4 M H_2SO_4, and the OD was measured in a spectrophotometer at an excitation wavelength of 492 nm.

BAL fibronectin content was measured by an ELISA procedure (Rennard *et al*., 1980). Briefly, 96-well plates were coated overnight at 4°C with a solution of 300 ng per well of fibronectin. BAL samples, at appropriate dilution, were incubated overnight at 4°C with a rabbit anti-fibronectin antibody (1/10,000, Anawa, Wangen, Switzerland) and then transferred to the fibronectin-coated wells. After washing the wells, the antibodies that did not react with fibronectin in the BAL samples were revealed by adding sequentially; a biotinylated secondary anti-rabbit antibody (1/100, Amersham, Little Chalfont, U.K.) and streptavidin horseradish peroxidase complex (1/1000, Amersham, Little Chalfont, U.K.). The substrate (2.2-Azino-bis (3-ethylbenzthiazole 6-sulphonic acid) diammonium, Sigma, St Louis, MO, U.S.A.) was then added for 5 min, and the reaction stopped with 10% SDS. The OD was measured at 405 nm.

Determination of total serum IgE level

Following the anaesthesia, blood was taken from the abdominal aorta, serum obtained and antibody titres determined by ELISA as described previously (Ledermann *et al*., 1991).

Measurement of ovalbumin-induced early bronchoconstriction

Airway response to ovalbumin was measured using barometric plethysmography using whole body plethysmography according to a previously published method (Hamelmann *et al*., 1997). For the acute challenge, conscious spontaneously breathing wild-type and Stat6-deficient mice were placed in a whole body plethysmograph and baseline airway function was measured, over 1 min, by calculating Penh. The animals were then exposed for 5 min to either 50 mg ml^{-1} of OVA in PBS or PBS only. Airway function was then measured for 20 min post-exposure. For the chronic challenge protocol, animals were exposed four times daily to OVA or PBS using the nose only exposure system, and the fifth challenge was carried out in the plethysmograph as described above for the acute challenge. The allergen induced early airway response was calculated by measuring the area under the per cent change in Penh $\times$ time curve post exposure. Microcal Origin software was used to calculate the area under the curve.

Data analysis

Data are expressed as mean $\pm$ s.e.mean. Statistical comparisons were performed using an ANOVA, and a P value of less than 0.05 was considered significant.

Results

No elevation of total serum IgE in the Stat6-deficient mice

As expected, wild-type OVA sensitized mice show increased levels of total serum IgE when compared with non-sensitized

animals (0.11 ± 0.02 μg ml^{-1}, $n=5$). Moreover, only chronic OVA-challenge induced a significant increase in total serum IgE levels, above the sensitization levels (Figure 1). By contrast, in both the acute and chronic challenge models and at all the time points studied, the levels of total serum IgE were below the limits of detection in Stat6-deficient mice (Figure 1).

Stat6 deficiency fully inhibits the development of BAL inflammation following acute challenge but only partially abrogate it following repeated challenges

When wild-type mice were challenged with a single OVA exposure, and groups of mice examined at various time-points up to 3 days, BAL showed sequentially, first a neutrophilic influx at day 1 and 2, followed by a massive eosinophilic cell infiltration concomitant with a small lymphocyte cell number increase at day 2 and 3. In contrast, none of these inflammatory cells were recruited in the BAL of Stat6-deficient mice following an acute OVA challenge, although a marginal and non-specific eosinophil cell number increase was observed at day 3 post challenge (Figure 2). In a second series of experiments, mice were repeatedly challenged for five consecutive days and sacrificed at different time points up to 7 days after the last challenge. In the wild-type mice, a significant BAL neutrophilia was observed at 5 h after the last challenge that resolved thereafter. BAL eosinophils were present at the first time point studied (5 h), peaked at day 3 and declined thereafter. BAL lymphocytes were present at all the time-points studied (Figure 3). In contrast to the situation observed after a single challenge, following multiple challenges Stat6-deficient mice were able to develop an airway inflammation with infiltration of neutrophils, eosinophils and lymphocytes into the BAL. The kinetics of inflammatory cell influx was similar to that observed in the wild-type animals, however, the intensity of the eosinophilic infiltration, was much less pronounced. In contrast, there was no difference in the intensity of the neutrophilic and lymphocytic infiltration between wild-type and Stat6-deficient mice (Figure 3). In both wild-type and Stat6-deficient mice, the macrophages numbers were not affected by the allergen challenge (data not shown). In both acute and repeated models, no BAL inflammation was observed in the wild-type and Stat6-deficient mice challenge with PBS where most of the cells were macrophages (Table 1). Acute OVA challenge of sham-sensitized mice (wild type and Stat6-deficient mice) failed to induce any accumulation of inflammatory cells (data not shown). Although neutrophils were recruited following repeated OVA challenges of sham-sensitized mice in both wild-type and Stat6-deficient mice (Table 2), greater numbers were present in the airways of OVA-sensitized mice (Figure 3). This neutrophilic influx probably reflects a non-specific inflammatory response following the introduction into the airways of a foreign protein.

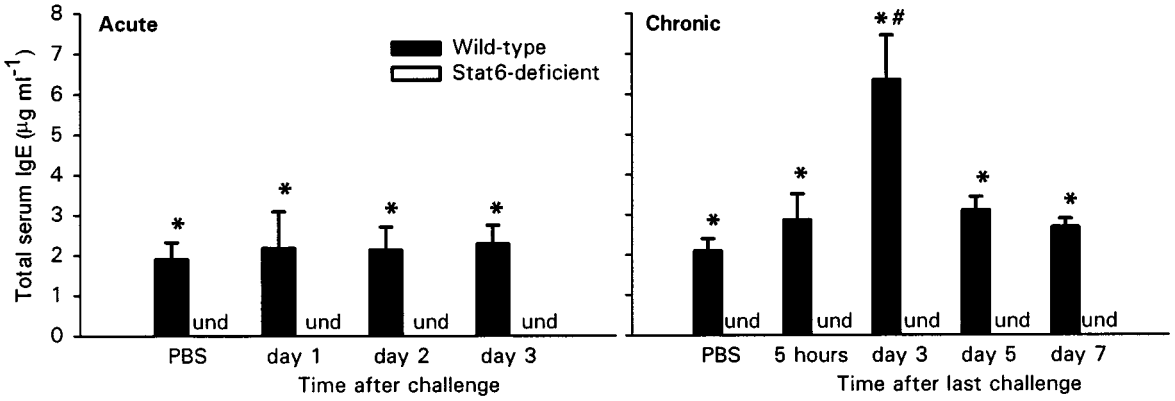

Figure 1 Time course of total serum IgE following acute and chronic OVA challenges in wild-type and Stat6-deficient mice. Animals were challenged once (acute) or challenged daily for five consecutive days (chronic) and then sacrificed at different time points following the last challenge. PBS-challenged mice were sacrificed 2 days after the last challenge for the acute model and 5 h after the last challenge for the chronic model. Data are expressed as mean$\pm$s.e.mean from six mice per groups. *$P<0.05$ versus Stat6-deficient mice. #$P<0.05$ versus PBS-challenged animals. und: undetectable.

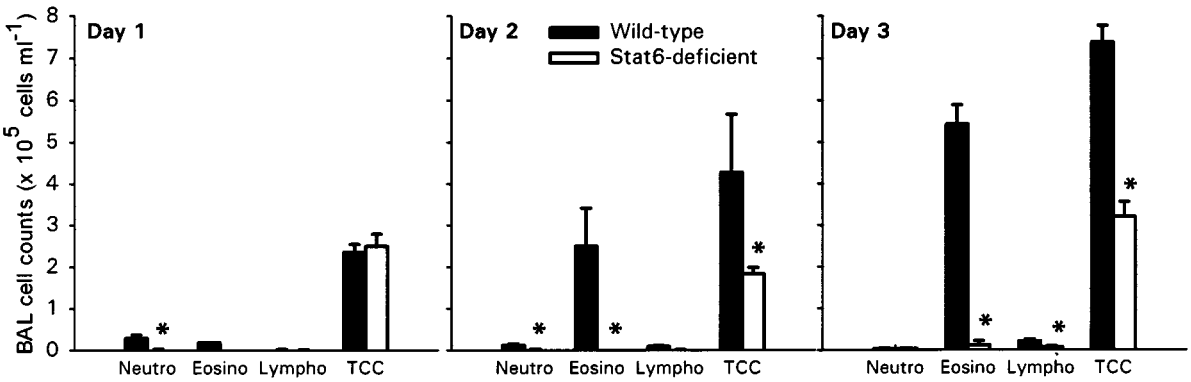

Figure 2 Time course of BAL inflammatory cell infiltration following acute OVA challenge. Wild-type and Stat6-deficient mice were challenged once with OVA and sacrificed at different time points after the challenge. Data are expressed as mean$\pm$s.e.mean from six mice per groups. *$P<0.05$ versus wild-type mice. Neutro: neutrophils, Eosino: eosinophils; Lympho, lymphocytes; TCC, total cell counts.

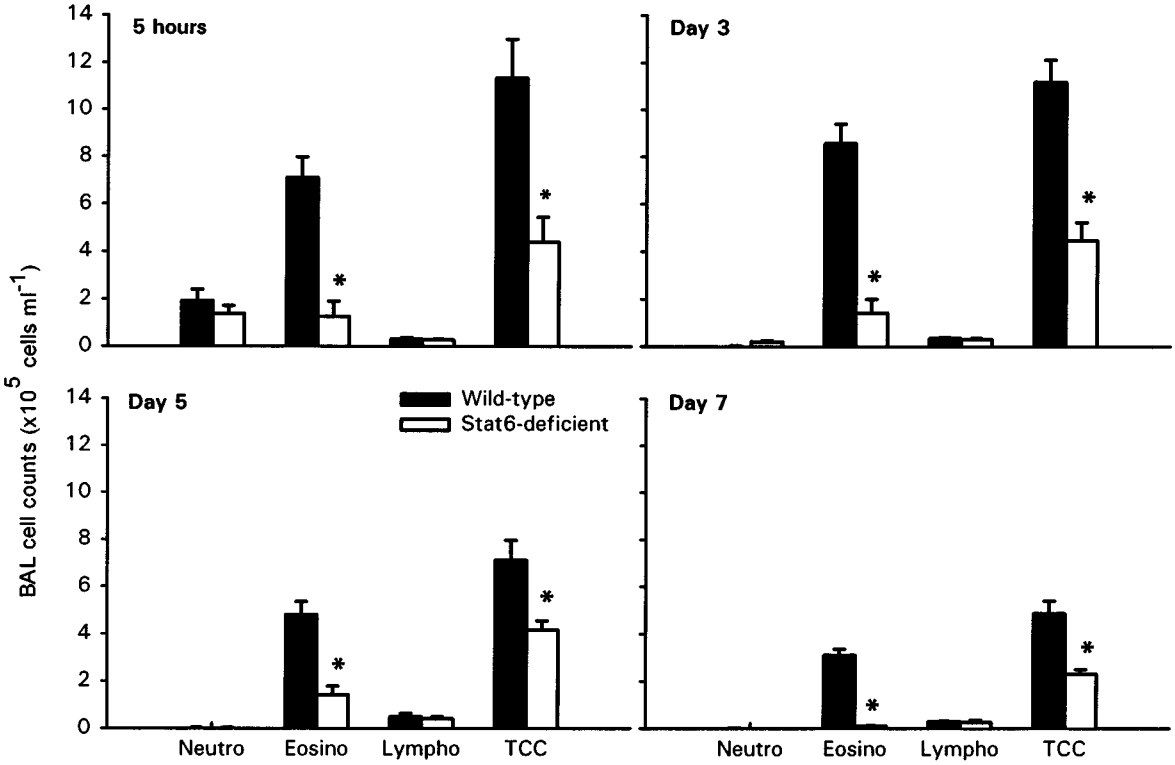

Figure 3 Time course of BAL inflammatory cell infiltration following chronic OVA challenge. Wild-type and Stat6-deficient mice were challenged daily with OVA, for five consecutive days and sacrificed at different time points after the last challenge. Data are expressed as mean ± s.e.mean from six mice per groups. $*P<0.05$ versus wild-type mice. Neutro: neutrophils, Eosino: eosinophils; Lympho, lymphocytes; TCC, total cell counts.

Table 1 Differential BAL cell counts following acute and chronic PBS challenges of sensitized wild-type and Stat6-deficient mice. Data are expressed as means ± s.e.mean of four animals per group

	Acute		*Chronic*	
	Wild-type ($\times 10^5$ cells ml^{-1})	*Deficient* ($\times 10^5$ cells ml^{-1})	*Wild-type* ($\times 10^5$ cells ml^{-1})	*Deficient* ($\times 10^5$ cells ml^{-1})
Macrophages	1.89 ± 0.32	2.47 ± 0.28	1.99 ± 0.21	2.57 ± 0.36
Lymphocytes	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00
Neutrophils	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.01
Eosinophils	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Total cell count	1.90 ± 0.35	2.50 ± 0.32	2.01 ± 0.24	2.60 ± 0.41

Sensitized animals were challenged once (acute) or daily challenged for five consecutive days (chronic) and BAL was performed 2 days after the last challenge for the acute model and 5 h after the last challenge for the chronic model.

Table 2 Differential BAL cell counts following repeated OVA challenges of sham-sensitized wild-type and Stat6-deficient mice. Data are expressed as means ± s.e.mean of five animals per group

	Wild-type ($\times 10^5$ cells ml^{-1})	*Deficient* ($\times 10^5$ cells ml^{-1})
Macrophages	2.23 ± 0.32	1.97 ± 0.13
Lymphocytes	0.01 ± 0.01	0.01 ± 0.01
Neutrophils	0.09 ± 0.02	0.13 ± 0.05
Eosinophils	0.00 ± 0.00	0.00 ± 0.00
Total cell count	2.31 ± 0.24	2.11 ± 0.41

Sham-sensitized animals were daily challenged for five consecutive days and BAL was performed 5 h after the last challenge.

To examine the activation state of BAL eosinophils, the EPO activity in the BAL supernatant was measured. No EPO activity could be detected following acute challenge in both wild-type and Stat6-deficient mice (data not shown). In contrast, following repeated challenges in wild-type mice, an increase in the EPO activity could be detected at 5 h and 3 days after the last challenge. An increase in BAL EPO was also detectable in Stat6-deficient mice, although it was smaller when compared with the wild-type animals (Figure 4). In both wild-type and deficient animals, no EPO activity could be detected from day 5 (data not shown). Background EPO activity was evident in both wild-type and deficient mice repeatedly challenged with PBS (Figure 4).

We then measured BAL fibronectin levels as a marker of plasma exudation. A time-dependent increase in BAL fibronectin levels was observed in the wild-type mice following both acute and chronic challenges when compared with PBS-challenged mice. However, this increase was much more pronounced after repeated challenge (Figure 5). In the Stat6-deficient mice, following acute challenge fibronectin could be detected in the BAL at all the time points studied, but this was not significantly increased when compared to PBS challenged animals. In contrast, following repeated challenges, a significant and sustained increase in BAL fibronectin levels was observed, although this increase was smaller when compared to the wild-type animals (Figure 5).

Stat6 deficiency fully inhibits the BAL Th2 cytokines levels following acute and repeated challenges

In wild-type mice, acute OVA challenge induced an increased production of IL-5 on day 1 and 2 post challenge; thereafter no detectable levels could be measured. IL-4 levels were increased only at day 1. In the Stat6-deficient mice, no IL-5 could be detected. IL-4 could be detected, however this was not significantly increased when compare to PBS challenged mice (Figure 6). Following repeated OVA challenges, wild-type mice showed a significant increase in IL-5 and IL-4 only at 5 h after the last challenge, when compared with PBS challenged mice. In contrast, OVA challenge of Stat6-deficient mice did not result in elevation of either IL-5 or IL-4, when compared with PBS challenged mice (Figure 6). In both models, no BAL IFN-γ could be detected in either the OVA and PBS challenged mice (data not shown).

Stat6 deficiency fully inhibits the allergen-induced bronchoconstriction following repeated challenges

In both wild-type and Stat6-deficient mice no bronchoconstriction was observed after an acute allergen challenge (data not shown). In contrast, immediately after the last of the chronic challenges, a bronchoconstrictive response was observed in the wild-type mice challenged with OVA when compared with wild-type mice challenged with PBS (Figure 7). By contrast, repeated OVA challenges did not induced any bronchoconstriction in the Stat6-deficient mice. There was no difference in baseline lung function, measured after PBS challenge, between wild-type and Stat6-deficient mice (Figure 7).

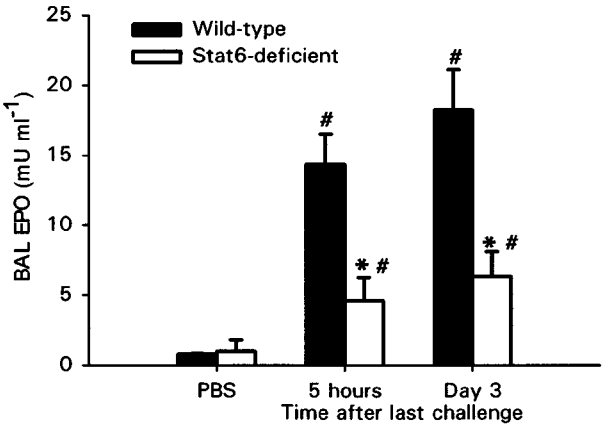

Figure 4 Time course of BAL EPO activity following chronic challenge. Wild-type and Stat6-deficient mice were challenged daily with OVA, for five consecutive days and sacrificed 5 h or 3 days after the last challenge. PBS-challenged mice were sacrificed 5 h after the last challenge. Data are expressed as mean ± s.e.mean from six mice per groups. *$P<0.05$ versus wild-type mice. #$P<0.05$ versus PBS-challenged animals.

Discussion

Recent studies using Stat6-deficient mice in an allergen driven model of lung inflammation have generated inconsistent results (Akimoto *et al*., 1998; Kuperman *et al*., 1998; Tomkinson *et al*., 1999). Although all these studies clearly outlined the role of Stat6 in the generation of IgE and in the development of airway hyperreactivity. Kuperman and colleagues demonstrated only a partial inhibition of BAL eosinophilia (Kuperman *et al*., 1998), whereas two other groups demonstrated almost complete inhibition of this parameter (Akimoto *et al*., 1998; Tomkinson *et al*., 1999). However, in these studies, the animal strains; the sensitization and challenge protocols were different. These differences may reflect the apparent discrepancies observed (Blyth *et al*., 1996; Zhang *et al*., 1997). In this investigation, we have used Stat6-deficient mice generated on a C57BL/6 background and compared the lung inflammatory response following single or repeated allergen challenges.

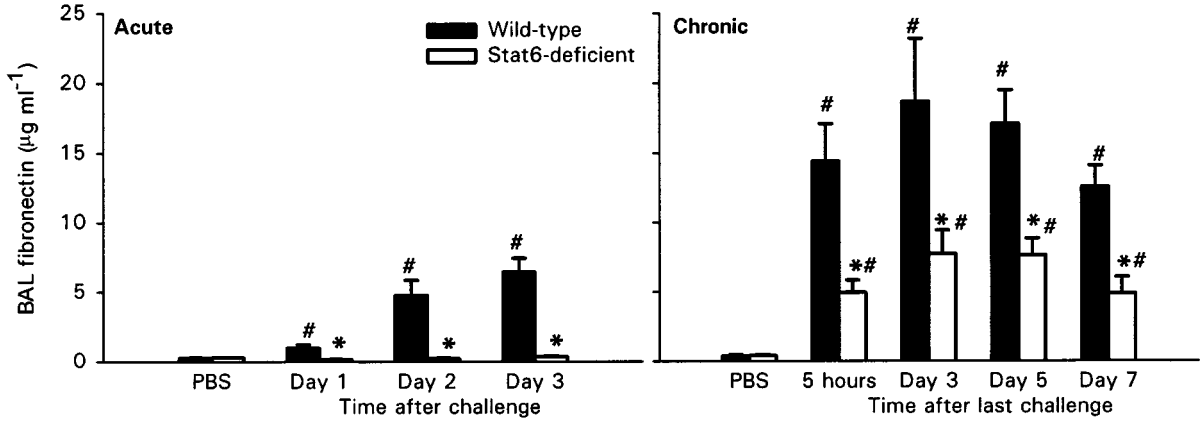

Figure 5 Time course of BAL fibronectin levels after acute and chronic challenges. Wild-type and Stat6-deficient mice were challenged once (acute) or challenged daily for five consecutive days (chronic) and then sacrificed at different time points after the last challenge. PBS-challenged mice were sacrificed 2 days after the last challenge for the acute model and 5 h after the last challenge for the chronic model. Data are expressed as mean ± s.e.mean from six mice per groups. *$P<0.05$ wild-type versus Stat6-deficient mice. #$P<0.05$ versus PBS-challenged animals.

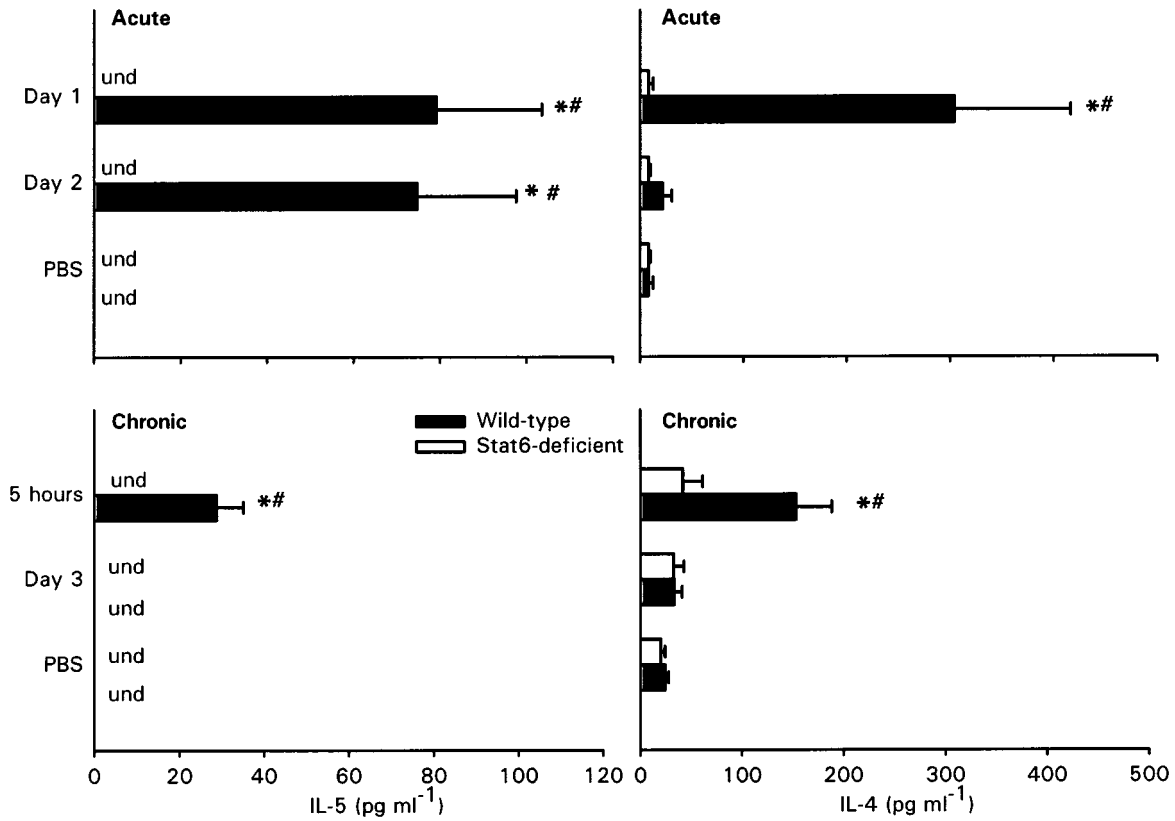

Figure 6 Time course of BAL cytokines levels after acute and chronic challenges. Wild-type and Stat6-deficient mice were challenged once (acute) or challenged daily for five consecutive days (chronic) and then sacrificed at different time points after the last challenge. PBS-challenged mice were sacrificed 2 days after the last challenge for the acute model and 5 h after the last challenge for the chronic model. Data are expressed as mean ± s.e.mean from six mice per groups. $*P<0.05$ wild-type versus Stat6-deficient mice. $\#P<0.05$ versus PBS-challenged animals. und: undetectable.

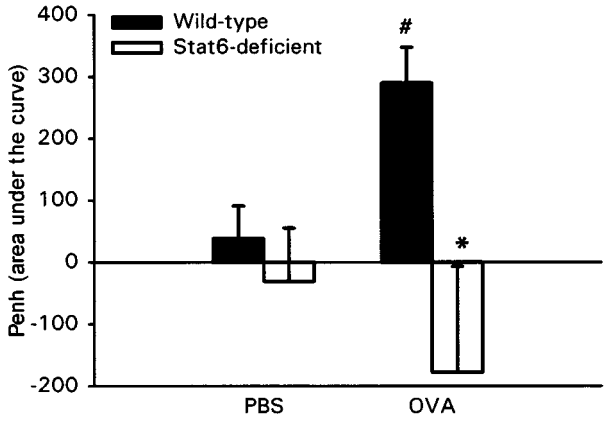

Figure 7 Allergen-induced bronchoconstriction after chronic challenges. Wild-type and Stat6-deficient mice were challenged daily for four consecutive days as described in Methods and the fifth challenge was carried out in a plethysmograph for 5 min. Airway function was then measured for 20 min post exposure. Data are expressed as area under the per cent change in Penh (mean ± s.e.mean) from 7–8 mice per groups. $*P<0.05$ wild-type versus Stat6-deficient mice. $\#P<0.05$ versus PBS-challenged animals.

In our model we investigated several important parameters observed in human asthma (Oettgen & Geha, 1999; Djukanovic *et al*., 1990). As such, following sensitization, wild-type mice exhibit elevated levels of serum IgE when compared to non-sensitized animals. Upon challenge, these animals develop an early bronchcoconstriction followed by a Th2 driven inflammation as demonstrated by the release of Th2 cytokines in the BAL (IL-4 and IL-5) and eosinophilic and lymphocytic infiltration of the airways. Th2 inflammation of the airways was also associated with an increase in plasma exudation, as judged by BAL fibronectin levels. There was no difference in the kinetics of these events between acute and chronic challenge but the intensity of these inflammatory parameters was much more pronounced following chronic challenge. By contrast, eosinophil activation, as measured by BAL EPO activity, an increase in total serum IgE over the sensitization level, and allergen-induced bronchoconstriction occurred only after chronic exposure to the allergen.

Airway hyperreactivity to exogenous stimuli is one of the major characteristics of asthma (Hargreave *et al*., 1982). However, the role played by IL-4 and IgE in the airway hyperreactivity is still controversial (Brusselle *et al*., 1995; Mehlhop *et al*., 1997; Hogan *et al*., 1997; Haile *et al*., 1999). The importance of Stat6 in this process was not investigated in the present study, but airway hyperreactivity has been previously shown to be totally abrogated in Stat6-deficient mice (Kuperman *et al*., 1998; Akimoto *et al*., 1998; Tomkinson *et al*., 1999). We studied allergen-induced bronchoconstriction, which is a process known to be primarily driven by IgE in human asthma (Oettgen & Geha, 1999). Despite the presence of serum IgE, a single allergen challenge was unable to induce bronchoconstriction in wild-type mice. When chronically challenged these mice developed a bronchoconstriction following the last challenge, which was associated with a further increase in serum IgE levels. It can be argued that the bronchoconstriction observed is related to the inflamed status of the lung. Our data has clearly demonstrated that Stat6-

deficient mice, resistant to bronchoconstriction and have no IgE, do develop subsequently lung inflammation comprising eosinophil activation and plasma leakage to about 50% of the levels observed in wild-type mice. These data, together with previously published work using neutralizing anti-IgE antibody (Haile *et al*., 1999) highlight the important role of IgE for the development of immediate bronchoconstriction to allergen in the murine model of lung inflammation.

In agreement with recently published studies, sensitized Stat6-deficient mice were unable to produce IgE and neither IL-4 or IL-5 levels could be increased following acute and chronic allergen challenge (Kuperman *et al*., 1998; Akimoto *et al*., 1998; Tomkinson *et al*., 1999). The lack of a Th2 response in Stat6-deficient mice is consistent with *in vitro* studies suggesting the requirement of Stat6 for IL-4-induced Th2 cell differentiation and immunoglobulin class switching to IgE (Shimoda *et al*., 1996; Kaplan *et al*., 1996). This is further supported by *in vivo* experiments using IL-4-deficient mice (Coyle *et al*., 1995; Brusselle *et al*., 1995) or neutralizing IL-4 antibody (Coyle *et al*., 1995) demonstrating the importance of IL-4 in the commitment of T cells to the Th2 phenotype.

Upon repeated challenge, we have clearly shown that, despite the lack of IgE and Th2 cytokines, Stat6-deficient mice were able to develop a lung eosinophilic inflammation of about 20% of that observed in wild-type mice. This strongly suggests that multiple mechanisms are likely to participate in allergen-induced lung eosinophilic recruitment depending on the challenge regimen. Thus, we can suggest that both Stat6-dependent and -independent mechanisms are involved in the development of lung eosinophilic inflammation following chronic challenge. Stat6-dependent inflammation is likely to be due to the role of IL-4 in the commitment of T cells to a Th2 phenotype as discussed above. We can only speculate about the Stat6-independent pathways, but inflammation in asthma and allergic disease is a complex phenomenon involving more than one signalling pathway. For example, the transcription factor nuclear factor κB (NF-κB) was shown to play an important role in the development of airway inflammation in murine model of asthma (Yang *et al*., 1998) and could overtake the absence of Stat6 signalling following chronic stimulation with the antigen.

In conclusion, our results confirm that Stat6 is an obligatory transcription factor for the synthesis of IgE following allergen exposure *in vivo*. Moreover, Stat6 signalling is also essential for the development of allergic airway inflammation following an acute allergen exposure. However, in a more chronic situation, the airway inflammatory response seems to be only partially mediated by Stat6. Since asthmatic patients are exposed chronically to natural allergens, our results are relevant to the strategies aimed at targeting the Stat6 pathway for the development of anti-asthma drugs.

References

AKIMOTO, T., NUMATA, F., TAMURA, M., TAKATA, Y., HIGASHIDA, N., TAKASHI, T., TAKEDA, K. & AKIRA, S. (1998). Abrogation of bronchial eosinophilic inflammation and airway hyperreactivity in signal transducers and activators of transcription (STAT)6-deficient mice. *J. Exp. Med.,* **187,** 1537–1542.

BERGSTEDT-LINDQVIST, S., MOON, H.B., PERSSON, U., MOLLER, G., HEUSSER, C. & SEVERINSON, E. (1988). Interleukin 4 instructs uncommitted B lymphocytes to switch to IgG1 and IgE. *Eur. J. Immunol.,* **18,** 1073–1077.

BLYTH, D.I., PEDRICK, M.S., SAVAGE, T.J., HESSEL, E.M. & FATTAH, D. (1996). Lung inflammation and epithelial changes in a murine model of atopic asthma. *Am. J. Respir. Cell Mol. Biol.,* **14,** 425–438.

BRUSSELLE, G., KIPS, J., JOOS, G., BLUETHMANN, H. & PAUWELS, R. (1995). Allergen-induced airway inflammation and bronchial responsiveness in wild-type and interleukin-4-deficient mice. *Am. J. Respir. Cell Mol. Biol.,* **12,** 254–259.

CASALE, T.B., WOOD, D., RICHERSON, H.B., ZEHR, B., ZAVALA, D. & HUNNINGHAKE, G.W. (1987). Direct evidence of a role for mast cells in the pathogenesis of antigen-induced bronchoconstriction. *J. Clin. Invest.,* **80,** 1507–1511.

COYLE, A.J., LE GROS, G., BERTRAND, C., TSUYUKI, S., HEUSSER, C.H., KOPF, M. & ANDERSON, G.P. (1995). Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am. J. Respir. Cell Mol. Biol.,* **13,** 54–59.

COYLE, A.J., WAGNER, K., BERTRAND, C., TSUYUKI, S., BEWS, J. & HEUSSER, C. (1996). Central role of immunoglobulin (Ig) E in the induction of lung eosinophil infiltration and T helper 2 cell cytokine production: inhibition by a non-anaphylactogenic anti-IgE antibody. *J. Exp. Med.,* **183,** 1303–1310.

DJUKANOVIC, R., ROCHE, W.R., WILSON, J.W., BEASLEY, C.R., TWENTYMAN, O.P., HOWARTH, R.H. & HOLGATE, S.T. (1990). Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.,* **142,** 434–457.

HAILE, S., LEFORT, J., EUM, S.Y., DUMAREY, C., HUERRE, M., HEUSSER, C. & VARGAFTIG, B.B. (1999). Suppression of immediate and late responses to antigen by a non-anaphylactogenic anti-IgE antibody in a murine model of asthma. *Eur. Respir. J.,* **13,** 961–969.

HAMELMANN, E., SCHWARZE, J., TAKEDA, K., OSHIBA, A., LARSEN, G.L., IRVIN, C.G. & GELFAND, E.W. (1997). Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.,* **156,** 766–775.

HARGREAVE, F.E., RYAN, G., THOMSON, N.C., O'BYRNE, P.M., LATIMER, K. & JUNIPER, E.F. (1982). Bronchial responsiveness to histamine or methacholine in asthma: measurement and clinical significance. *Eur. J. Respir. Dis.,* **121,** 79–88.

HOGAN, S.P., MOULD, A., KIKUTANI, H., RAMSAY, A.J. & FOSTER, P.S. (1997). Aeroallergen-induced eosinophilic inflammation, lung damage, and airways hyperreactivity in mice can occur independently of IL-4 and allergen-specific immunoglobulins. *J. Clin. Invest.,* **99,** 1329–1339.

IHLE, J.N. (1995). Cytokine receptor signalling. *Nature,* **377,** 591–594.

KAPLAN, M.H., SCHINDLER, U., SMILEY, S.T. & GRUSBY, M.J. (1996). Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity,* **4,** 313–319.

KORSGREN, M., ERJEFALT, J.S., KORSGREN, O., SUNDLER, F. & PERSSON, C.G. (1997). Allergic eosinophil-rich inflammation develops in lungs and airways of B cell-deficient mice. *J. Exp. Med.,* **185,** 885–892.

KUPERMAN, D., SCHOFIELD, B., WILLS-KARP, M. & GRUSBY, M.J. (1998). Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J. Exp. Med.,* **187,** 939–948.

LEDERMANN, F., SCHLIENGER, C., WAGNER, K. & HEUSSER, C. (1991). A sensitive and efficient induction system for murine IgE. Single cell analysis at the clonal level. *J. Immunol. Methods,* **141,** 263–275.

MEHLHOP, P.D., VAN DE RIJN, M., GOLDBERG, A.B., BREWER, J.P., KURUP, V.P., MARTIN, T.R. & OETTGEN, H.C. (1997). Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 1344–1349.

OETTGEN, H.C. & GEHA, R.S. (1999). IgE in asthma and atopy: cellular and molecular conections. *J. Clin. Invest.,* **104,** 829 – 835.

RENNARD, S.I., BERG, R., MARTIN, G.R., FOIDART, J.M. & ROBEY, P.G. (1980). Enzyme-linked immunoassay (ELISA) for connective tissue components. *Anal. Biochem.,* **104,** 205 – 214.

ROBINSON, D.S., HAMID, Q., YING, S., TSICOPOULOS, A., BARKANS, J., BENTLEY, A.M., CORRIGAN, C., DURHAM, S.R. & KAY, A.B. (1992). Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.,* **326,** 298 – 304.

SHIMODA, K., VAN DEURSEN, J., SANGSTER, M.Y., SARAWAR, S.R., CARSON, R.T., TRIPP, R.A., CHU, C., QUELLE, F.W., NOSAKA, T., VIGNALI, D.A., DOHERTY, P.C., GROSVELD, G., PAUL, W.E. & IHLE, J.N. (1996). Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature,* **380,** 630 – 633.

SWAIN, S.L., WEINBERG, A.D., ENGLISH, M. & HUSTON, G. (1990). IL-4 directs the development of Th2-like helper effectors. *J. Immunol.,* **145,** 3796 – 3806.

TOMKINSON, A., KANEHIRO, A., RABINOVITCH, N., JOETHAM, A., CIESLEWICZ, G. & GELFAND, E.W. (1999). The failure of STAT6-deficient mice to develop airway eosinophilia and airway hyperresponsiveness is overcome by interleukin-5. *Am. J. Respir. Crit. Care Med.,* **160,** 1283 – 1291.

WALKER, C., VIRCHOW, J.C.J., BRUIJNZEEL, P.L. & BLASER, K. (1991). T cell subsets and their soluble products regulate eosinophilia in allergic and nonallergic asthma. *J. Immunol.,* **146,** 1829 – 1835.

YANG, L., COHN, L., ZHANG, D.H., HOMER, R., RAY, A. & RAY, P. (1998). Essential role of nuclear factor kappaB in the induction of eosinophilia in allergic airway inflammation. *J. Exp. Med.,* **188,** 1739 – 1750.

ZHANG, Y., LAMM, W.J., ALBERT, R.K., CHI, E.Y., HENDERSON, W.R.J. & LEWIS, D.B. (1997). Influence of the route of allergen administration and genetic background on the murine allergic pulmonary response. *Am. J. Respir. Crit. Care Med.,* **155,** 661 – 669.

(Received February 8, 2000
Revised May 16, 2000
Accepted May 23, 2000)