

## Inhibition of stem cell factor reduces pulmonary cytokine levels during allergic airway responses

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### SUMMARY

Stem cell factor (SCF) has a significant role in the inflammation and activation of allergic airway responses. When monoclonal anti-SCF was administered intratracheally during allergen challenge there was a significant alteration of eosinophil accumulation and airway hyperreactivity (AHR). Anti-SCF treatment also attenuated pulmonary cytokine and chemokine levels. In particular, there was an antibody dose-dependent decrease in interleukin (IL)-5 and tumour necrosis factor (TNF)- $\alpha$ . There was also a significant reduction of CCL2 and CCL5, which correlated with the reduction in AHR. Mice treated with anti-SCF demonstrated a significant decrease in pulmonary *gob-5* gene expression, which has been shown to correlate to goblet cell hyperplasia/metaplasia relating to airway mucus production. Blocking SCF-mediated activation within the airway using a monoclonal antibody indicates that this cytokine may represent a viable target for therapeutic intervention that could affect multiple aspects of allergen-induced immunopathology.

**Keywords** asthma cytokines mucus stem cell factor

### INTRODUCTION

The accumulation and activation of leucocytes within the airway plays a central role in the exacerbation of severe asthmatic responses [1]. Recent studies have identified that stem cell factor (SCF) induces a number of responses within the airway that result in increased severity of the airway responses. SCF is identified predominantly as an important haematopoietic factor that has a critical role in the maturation of numerous leucocyte populations as well as erythrocytes [2,3]. In addition, SCF is a terminal differentiation factor for mast cells and inhibits their apoptosis in peripheral tissues [4–9]. Animals deficient in c-kit (SCF receptor; WWv) function or mice that have decreases in SCF (Sld) demonstrate a paucity of peripheral mast cells [7,10,11]. Thus, SCF signals are required for populating and maintaining the peripheral tissues with these important sentinel leucocytes.

SCF has been found to be expressed in nearly every cell population that has been examined. SCF initially presents itself as a membrane form and during inflammatory/immune responses proteases cleave the cytokine from the cell surface [11,12]. There is also a truncated form of SCF that lacks the cleavage site and cannot be solubilized from the surface of the cell. Both the soluble

and membrane forms of SCF are involved in cellular activation through a tyrosine kinase mediated signalling cascade. The ability of SCF to activate effector leucocytes directly, including mast cells and eosinophils, may be central to its involvement in inflammatory responses. Data have accumulated on the importance of SCF-induced activation for mast cells, related not only to allergic responses, but also during initial bacterial-induced responses [13]. Possibly the most important aspect of SCF is its ability to directly activate local mast cell populations within the airway [14–17]. Studies have demonstrated the exogenous administration of SCF can induce adverse airway activation significantly, resulting in hyperresponsiveness in the absence of any other stimuli. This appears to be related to induction and release of cystienyl leukotrienes within the lungs that have been associated closely with the induction of severe asthmatic responses. In addition, several studies have now demonstrated that inhibition of SCF clearly attenuates the severity of the allergen-induced responses in murine models of airway disease [14,16–19]. Finally, the identification of SCF as an eosinophil-activating factor may broaden the role of SCF in chronic asthmatic responses [20,21].

In the present studies we have extended observations on the role of SCF during allergic responses by demonstrating that a monoclonal antibody specific for SCF alters the airway responses by reducing both activating [tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-5] as well as chemotactic [monocyte chemoattractant protein (MCP)-1, regulated upon activation normal T cell expressed and secreted (RANTES)] cytokine production in a

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dose dependent manner. The reduction in these responses results in significant reduction in eosinophil accumulation and airway hyperreactivity.

## MATERIALS AND METHODS

### Mice

CBA/J mice were purchased from Jackson Laboratory and maintained under specific pathogen-free conditions.

### Cockroach antigen challenge

Allergic mice were immunized with cockroach allergen (Bayer Corporation, Elkhart, IN, USA) as described previously [22–24]. Briefly, mice were immunized with 10 µg of cockroach allergen in incomplete Freund's adjuvant (IFA) on day 0. On day 14 the mice were given an intranasal challenge of 10 µg of cockroach allergen in 10 µl of diluent to localize the response to the airway. This initial intranasal challenge with antigen induced little cellular infiltrate into the lungs of the mice upon histological examination. Mice were then rechallenged 6 days later by intratracheal administration of 10 µg of cockroach allergen in 50 µl of sterile phosphate buffered saline (PBS) or with PBS alone (vehicle) and given a second challenge of cockroach allergen 48 h after the first intratracheal challenge. Along with the intratracheal injections of cockroach allergen, mice were given either anti-SCF (monoclonal R&D Systems, Rochester, MN, USA) or control antibody treatment (100 µg).

### Measurement of airway hyperreactivity

Airway hyperreactivity in anaesthetized mice was measured as previously described with a mouse plethysmograph (Buxco, Troy, NY, USA) using a direct ventilation method designed specifically for low tidal volumes [22–24]. A single optimal methacholine dose (125 µg/kg) was used that elicited a minimal response in control CBA/J mice but gave a significant hyperreactive response in allergic mice. There was no difference in background methacholine responses in anti-SCF versus control treated CBA/J mice (data not shown).

### Enzyme linked immunosorbent assays (ELISAs)

Whole lungs were homogenized in 1 ml of lysis buffer (PBS with 0.01% triton X-100 non-ionic detergent) containing protease inhibitors. Debris-free supernatants were isolated and the cytokines measured by ELISA as described [25,26]. Antibody pairs from R&D Systems were used for ELISAs. The sensitivity of the analyses was ~10 pg/ml. No cross-reactivity to any other chemokine or cytokine was detected in individual assays.

### Quantitative polymerase chain reaction (PCR) analysis of gob-5

RNA was purified from whole lung homogenates using TRIzol reagent (GIBCO, Grand Island, NY, USA) and chloroform. RNA was quantified by measuring absorbance at 260 nm. Samples were then standardized to 5 mg/ml with DEPC water. RNA was then reverse transcribed to cDNA and 1 µl of this cDNA was used in the TaqMan reaction mixture. The specific primer/probe sets for real-time PCR were predeveloped by Applied Biosystems. Briefly, during PCR a fluorogenic probe consisting of an oligonucleotide with both a reporter and a quencher dye attached anneals specifically between the forward and reverse primers. When the probe is cleaved by the 5' nuclease activity of the DNA

polymerase, the reporter dye is separated from the quencher dye and a sequence-specific signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored during the PCR. This real-time detection generates quantitative data based on the PCR at early cycles when PCR fidelity is the highest.

### Statistics

Statistical significance was determined by ANOVA and different sets were further examined by Student's *t*-test. *P*-values less than 0.05 were considered significant.

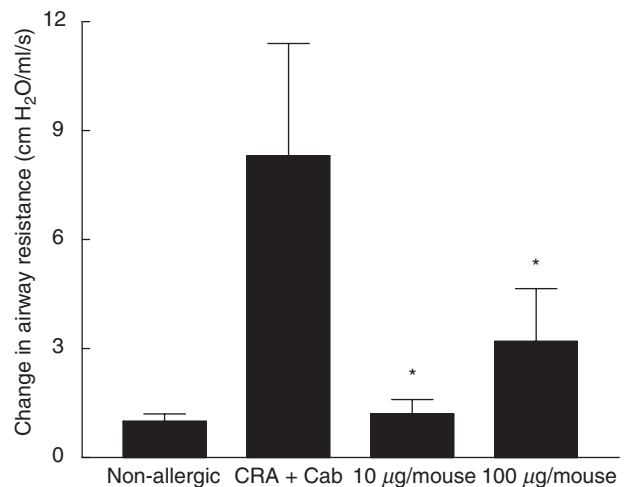
## RESULTS

### Monoclonal anti-SCF blocks airway hyperreactivity and eosinophilia after multiple allergen challenges

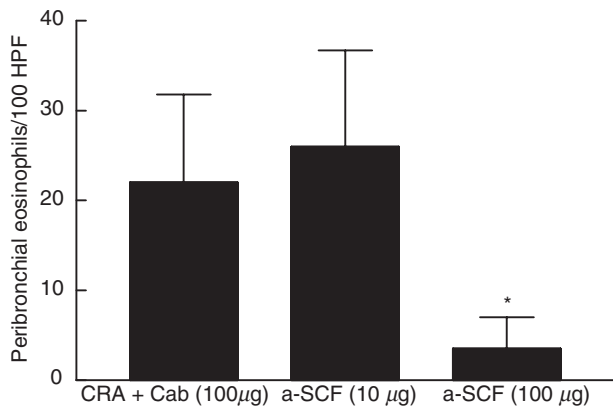
We have demonstrated previously that SCF-mediated responses have a role in the development of airway hyperreactivity (AHR) after an allergen challenge [16,17]. The data in Fig. 1 extend the previous findings by showing that by inhibiting SCF using a monoclonal anti-SCF antibody during multiple allergen challenges a significant reduction in AHR can be achieved with both doses of anti-SCF treatment. In contrast, the peribronchial eosinophil accumulation was quite different as the higher dose of anti-SCF reduced eosinophil accumulation, whereas the lower dose did not (Fig. 2). Thus, these studies with a monoclonal antibody verify earlier observations using polyclonal antibody therapy in the airway but also demonstrate that SCF may have multiple roles in the responses driving both the inflammatory response as well as the hyperreactivity responses.

### Monoclonal antibody treatment attenuates cytokine and chemokine levels after cockroach allergen challenges

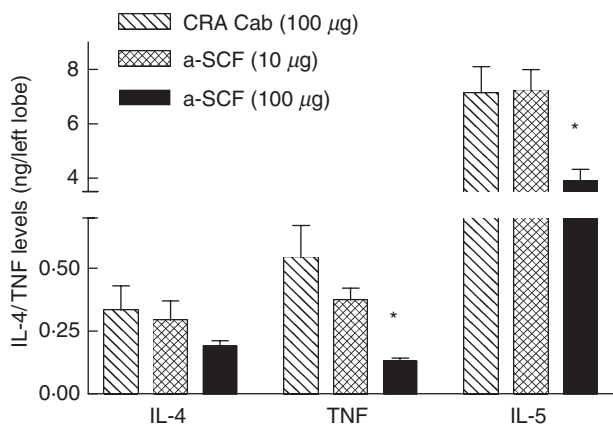
Inhibition of SCF in the airway may have a significant impact on the overall inflammatory response including the production of



**Fig. 1.** Treatment of animals with anti-SCF into the airway attenuates hyperreactivity. Two doses of anti-SCF (10 and 100 µg/mouse) was given to individual groups at the time of intratracheal allergen challenge. Twenty-four hours after final allergen challenge, animals are examined for changes in airway resistance. Both concentrations of antibody significantly reduced airway resistance but were not different from one another. Data represent mean ± s.e. of six mice/group. \**P* < 0.05 compared to the control antibody (Cab) CRA group.

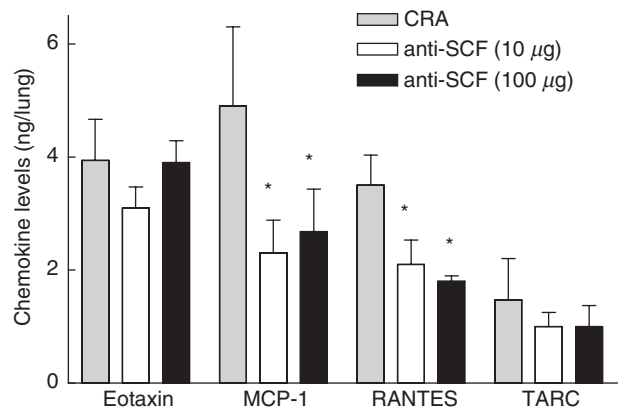


**Fig. 2.** Accumulation of peribronchial eosinophils is attenuated by the highest dose of anti-SCF treatment (100 µg/mouse). Two doses of anti-SCF (10 and 100 µg/mouse) were given to individual groups at the time of intratracheal allergen challenges. Twenty-four hours after allergen challenge, animals were sacrificed and the right lobe of lungs were harvested and processed histologically. Morphometric enumeration of eosinophils was performed on differentially stained serial sections by examining 100 high-powered fields (HPF; 1000× mag)/mouse. Data represent mean ± s.e.m. from six mice in each group. \**P* < 0.05 compared to the control antibody CRA group.



**Fig. 3.** Neutralization of SCF in airways of cockroach antigen-challenged allergic mice reduces cytokine levels. Twenty-four hours after allergen challenge left lobe of lungs were harvested and processed for specific ELISAs in 1 ml of prepared buffer. Data represent mean ± s.e.m. from six mice in each group. \**P* < 0.05 compared to the control antibody CRA group.

cytokines and chemokines. Cytokine and chemokine levels in the lungs of animals treated with anti-SCF antibody were compared to control treated animals. The data demonstrated that by blocking SCF a significant decrease in IL-5 and TNF-α was observed at the higher dose of anti-SCF (100 µg/mouse), whereas IL-4 was reduced but did not reach significance (Fig. 3). Examination of specific chemokines that have been implicated in allergen-induced responses indicated that both MCP-1 and RANTES levels were significantly altered whereas eotaxin and TARC were not significantly reduced (Fig. 4). These data demonstrated that anti-SCF treatment attenuated both cytokines and chemokines that have been associated with allergen-induced airway hyperreactivity.



**Fig. 4.** Reduced levels of chemokines in allergen-challenged animals treated with anti-SCF. Twenty-four hours after allergen challenge left lobe of lungs were harvested and processed for specific ELISAs in 1 ml of prepared buffer. Data represent mean ± s.e.m. from six mice in each group. \**P* < 0.05 compared to the control antibody CRA group.

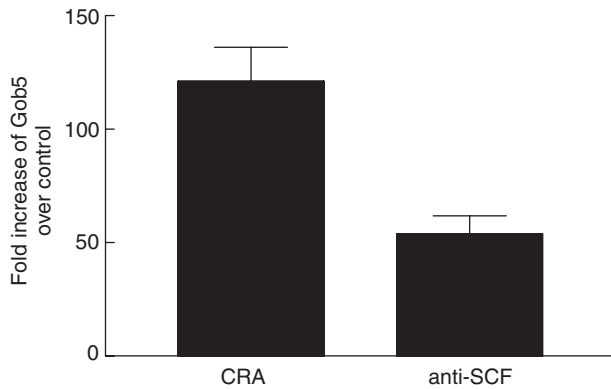
*Inhibition of SCF in the airway reduces the mucus-related gene expression, gob-5 (mclca3)*

A significant pathophysiological aspects of asthma that can be detrimental during an induced response is the activation and overproduction of mucus. Recent studies have identified a protein that regulates goblet cell maturation and mucus overproduction, gob-5 (mCLCA3), and is expressed in human asthma [27,28]. In the present studies, when we examined the expression of gob-5 we found a significant decrease in gene expression using real-time PCR analysis (Fig. 5). To determine if the gob-5 expression reflected goblet cell presence in the airways of the allergen-challenged mice, histological sections were stained with PAS/alcian blue to identify mucus producing goblet cells in the airway. Figure 6 illustrates that the control antibody-treated animals exhibited a rather intense expression of mucus. Although the anti-SCF-treated animals exhibited some mucus positive staining airway cells, the intensity was considerably less throughout the lung. These responses demonstrate that SCF may initiate a broad array of detrimental responses during the initiation and maintenance of an allergic airway response.

**DISCUSSION**

The identification of viable targets for therapy of asthmatic disease has centred in recent years on blocking inflammatory responses and reducing infiltration and activation of leucocytes. Previous studies that have examined the role of SCF established that SCF is produced during allergen-induced responses and when neutralized alters airway function [14,16,17]. We have extended our previous observations on the role of SCF in allergic airway disease by demonstrating that by blocking SCF locally in the airway a reduction of a number of crucial cytokines and chemokines related to the immunopathology can be altered. The cytokines that were reduced significantly by inhibiting SCF were TNF-α and IL-5. Both of these cytokines have demonstrated a significant role in the exacerbation of asthmatic inflammation [29–31]. TNF-α has been shown to influence detrimental changes in airway physiology both directly as well as indirectly by inducing chemokines, such as RANTES and MCP-1 [32–34]. Interestingly, although the lower dose of anti-SCF was able to attenuate AHR,

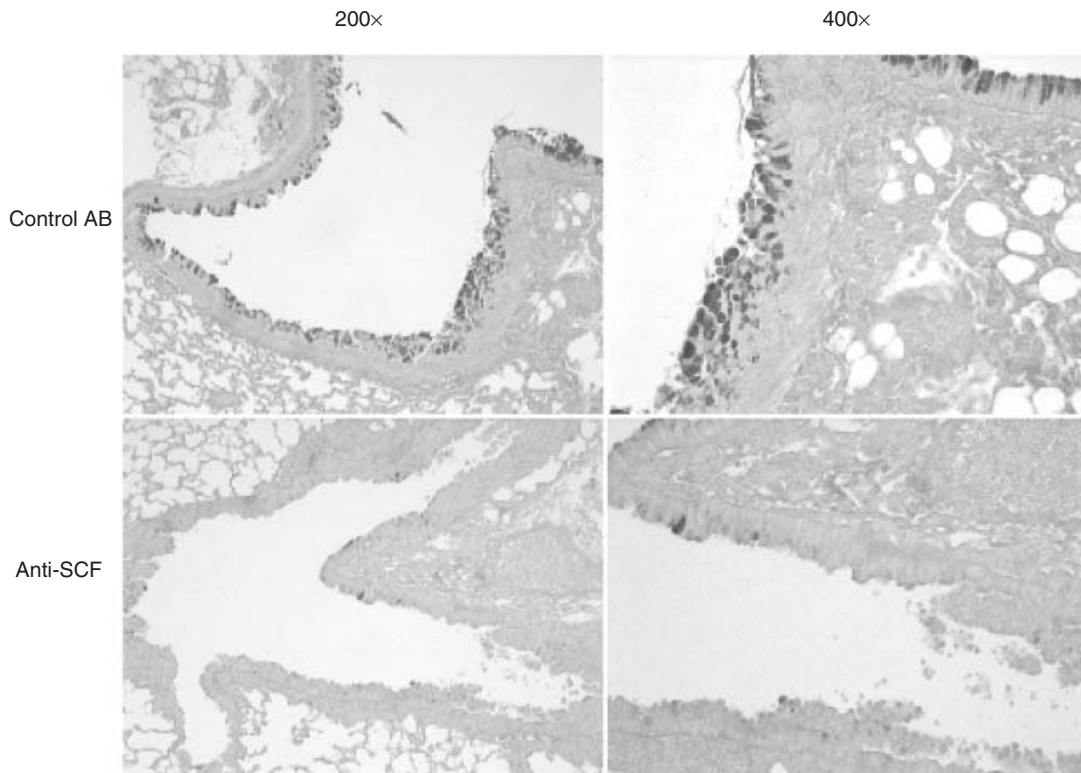
there was no reduction in peribronchial eosinophils. The primary role of IL-5 has clearly been linked to the maturation and migration of eosinophils during allergic responses [35–37], IL-5 and eosinophil accumulation were reduced only with the highest level of anti-SCF. It may be that the lower level of anti-SCF was suffi-



**Fig. 5.** Anti-SCF treatment reduces the expression of *gob-5* mRNA. In separate studies, whole lung mRNA was isolated from unchallenged, cockroach allergen challenged with control antibody (CRA) or anti-SCF (100  $\mu$ g) monoclonal antibody-treated and allergen-challenged animals. Reverse transcribed cDNA from whole lungs was subjected to *gob-5* specific real-time PCR analysis. Data represent the fold increase in expression in CRA + control antibody or CRA + anti-SCF treated *versus* unchallenged allergic animals. Data represent mean  $\pm$  s.e. from four mice in each group. \* $P < 0.05$  compared to the control antibody CRA group.

cient to reduce release of other bronchospastic mediators, such as leukotriene production, but not eosinophilia. Thus, higher levels of anti-SCF appear to attenuate better the eosinophil accumulation. There have now been multiple publications that have separated eosinophils and airway hyperreactivity in animal models [38,39]. However, eosinophil involvement in airway function may be more dependent upon activation and degranulation of eosinophils and not their mere presence, an aspect where SCF has been implicated [21]. The reduction of MCP-1 and RANTES with both doses of anti-SCF correlated well with the reduced hyperreactivity responses. Both of these chemokines have been implicated coincidentally in asthmatics with severe disease and have demonstrated further significant roles in animal models of airway disease [32,40–47]. Interestingly, SCF has been shown to induce TNF- $\alpha$ , MCP-1 and RANTES production from mast cells [48–50], suggesting that these sentinel cells may be affected directly by locally released SCF within the lung. In addition, recent studies in our lab have identified that SCF-stimulated eosinophils also produce significant levels of chemokines [21], including RANTES. Thus, it may be that these chemokines play an important role in the generation of the AHR, as they correlated well with the reduction in the AHR within the treated animals.

Surface-bound SCF can be quickly cleaved and released during inflammatory responses and react with surrounding cell populations [4,51–53]. Because SCF is not secreted immediately but is typically displayed first on the surface of a number of structural and inflammatory cell populations, there is probably a considerable ‘reservoir’ of SCF within the lung. Previous data from our laboratory has identified ~20 ng of total (bound and unbound) SCF/



**Fig. 6.** The expression of mucus staining within the airway epithelium was reduced by treatment of allergen-challenged mice with anti-SCF compared to control treated animals. The histological sections were stained with PAS-alcian blue stain to identify the mucus producing cells within the airways of challenged mice. Naive animals have no positive staining in their airways (not shown).

lung in naive mice that is significantly up-regulated during allergen challenge [14]. Thus, the ability to quickly release the surface bound SCF may serve as a critical step in the progression of the allergic asthmatic responses. Further studies have demonstrated that SCF can be produced in peribronchial myofibroblasts and smooth muscle cells [54–57]. Blockade of the SCF-c-kit activation pathway may also be a viable means of altering the progression of these responses. In addition to the reduction of the inflammatory responses the present studies also highlight the role of SCF in the reduction of *gob-5* gene (mCLCA3) and airway mucus expression. *gob-5* has been shown to play a significant role in the generation of mucus in studies that have either overexpressed or blocked expression of *gob-5* [27]. Although it is not completely clear how *gob-5* relates to mucus production it does appear that goblet cell hyperplasia and mucus production is closely related to *gob-5* expression as exhibited in these studies. This is also true of its human homologue CLCA1, which is highly up-regulated in asthma patients and implicated in cystic fibrosis [27,58–60]. These data together support the notion that blocking SCF-mediated pathways may be beneficial for altering the airway responsiveness and detrimental pathology that develops during asthmatic airway responses.

The reduction in mast cell activation and decrease in eosinophils in animals treated with anti-SCF during the allergic airway response has been shown to correlate with the alteration of pathophysiology [14,16]. Interestingly, SCF has been shown to directly activate eosinophils for increased migration [20]. Possibly more important to the severity of allergic disease is the recent observation that SCF can induce eosinophil activation, degranulation and chemokine production directly [21]. Thus, this latter set of studies further broadens the scope of the biological effect of SCF function during allergic airway responses. Although we did not measure parameters of mast cell and eosinophil activation directly within these studies, it may be that the lower dose of anti-SCF was sufficient to block some aspects of mast cell and eosinophil activation, such as degranulation, while reducing the induction of inflammatory mediators incompletely, such as the chemokines. It is difficult to tell from these studies whether the SCF is involved directly or indirectly in the production of these mediators; however, probably both apply.

The data in the present studies extend further our observations on the role of SCF during allergic airway responses via the ability to regulate particular cytokines and chemokines. In addition, the reduced inflammation appears to relate directly to the alteration in mucus-related, *gob-5* gene expression. These data, along with previous observations of the role of SCF, contribute to the notion that SCF has an important role on the development of detrimental airway responses during allergen-induced disease.

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