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Stem Cell Factor-Induced Airway Hyperreactivity in Allergic and Normal Mice

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The induction of airway hyperreactivity during allergic responses involves multiple ill-defined mechanisms. Recently a role for stem cell factor (SCF) in the development of allergic eosinophilic airway inflammation has been identified. In the present study we demonstrate that SCF has a role in both the inflammatory response and airway hyperreactivity. Neutralization of SCF or examination of SCF-mutant mice, which were deficient in SCF and pulmonary mast cells, demonstrated significant alterations in the allergen-induced airway hyperreactive responses. The reduced hyperreactivity response was accompanied by a significant reduction in eosinophil accumulation. To examine the direct role of SCF on airway hyperreactivity, we administered SCF into the airways of normal mice via intratracheal injections and demonstrated a dose dependent increase in airway hyperreactivity at 4 hours that was maintained at 24 hours after administration. Instillation of SCF into SCF-deficient (mast cell deficient) mice demonstrated significantly lower increases in airway hyperreactivity compared with the littermate controls with normal mast cell numbers. These studies demonstrate that locally expressed SCF can induce changes in airway physiology via mast cell activation, verifying the role of SCF in allergic airway inflammation and hyperreactivity. ***(Am J Pathol 1999, 154:1259–1265)***

Despite continued efforts to understand the mechanisms that drive airway responses, morbidity because of asthma continues to rise.[1,2] The initiation and maintenance of allergic airway inflammation is mediated by multiple mechanisms. The design of specific therapeutic intervention in this disease is difficult. Therefore, it is important to identify novel mechanisms of activation and regulation that can lead to new therapeutics. Peribronchial leukocyte accumulation is the hallmark of asthma.[3–9] In particular, eosinophils have been reported to be the primary cell associated with induction of bronchial mucosal injury and are thought to participate in bronchial obstruction and airway hyperreactivity.[6,7] However, other cell populations within the lung, such as mast cells, must be considered as important populations that may initiate and directly contribute to airway damage and hyperreactivity.[8,9] Several therapeutic strategies have focused on attenuating airway inflammation, including glucocorticoids, cromolyn sodium, and other agents that nonspecifically affect the response.[10] The limited therapeutic options for the treatment of the disease likely reflect the lack of our understanding of the mechanisms that cause airway inflammation and hyperreactivity.

The major pathophysiological event that occurs during asthma is airway hyperreactivity during the late phase response. The initial induction of IgE-mediated mast cell degranulation constitutes the primary mechanism that drives the allergic response and lends to both the early and late phase changes in airway physiology.[3–9] In addition to IgE-mediated mechanisms, it appears that c-kit ligand or stem cell factor (SCF) can directly induce mast cell activation as well as augment the IgE-mediated response. The prolonged activation of local airway mast cell populations by SCF after initial IgE-mediated events may play a significant role in persistent activation leading to a late phase response. SCF is not only an important hematopoietic factor that drives terminal differentiation of mast cells, but it has been shown to have other important roles in regulating mast cell biology such as survival, activation, and degranulation of mature mast cells.[11–16] SCF has also demonstrated a direct role on eosinophil adhesion by altering the avidity of VLA-4 on the surface of the eosinophil.[17,18] Previous data indicates that SCF has an important role during allergen-[19] and parasite-[20] driven responses and contributes to eosinophil accumulation. In addition, SCF has been shown to directly stimulate mast cell activation in human bronchi and induce smooth muscle cell contraction.[21] SCF not only enhances histamine release but also appears to induce leukotriene release from mast cells.[22] Thus, SCF may have both direct and indirect roles in mediating airway inflammation and hyperreactivity.

The results from the present studies indicate that SCF has a role in the induction of airway hyperreactivity during allergic responses and can directly induce airway hyperreactivity when injected into the airways of normal mice.

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The reduction of allergic airway hyperreactivity in the absence of SCF appears to correlate directly to the accumulation of eosinophils. In contrast, the direct induction of airway hyperreactivity in normal mice appears to be centered around mast cell activation as mast cell-deficient mice (SCF deficient) have a significantly altered hyperreactive response.

Materials and Methods

Animals

Female CBA/J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were maintained under standard pathogen-free conditions.

Egg Isolation and Soluble Egg Antigen Protein Preparation

Soluble egg antigens (SEA) were prepared from acutely *Schistosoma mansoni*-infected mice as previously described.[23] Briefly, eggs were isolated from livers of infected mice after a 3-day incubation and ground on ice to release the soluble antigens from the egg. The preparation was then spun in an ultracentrifuge at 100,000 $\times$ *g* for 2 hours, and the supernatant was collected.

Sensitization and Induction of the Airway Response

To induce a Th2-type response, the following procedure was established in normal CBA/J mice.[23] Briefly, the mice were immunized with 5000 isolated *S. mansoni* eggs intraperitoneally at days 0 and 7 of the protocol. On day 14 the mice were given an intranasal challenge of 10 μg of SEA in 10 μl of phosphate-buffered saline (PBS) to localize the response to the airway. This initial intranasal challenge with antigen induced little cellular infiltrate into the lungs of the mice on histological examination. Mice were then rechallenged 6 days later by intratracheal administration of 10 μg of SEA in 25 μl of sterile PBS or with PBS alone (vehicle). The magnitude of infiltration in both the vehicle control and SEA-challenged mice was examined histologically. Only the SEA-challenged mice displayed a significant inflammatory response that included eosinophil infiltration, as previously described.[23]

Morphometric Analysis of Peribronchial Eosinophils

Lungs from mice immunized and challenged with SEA or saline vehicle were preserved with 1 ml of 4% paraformaldehyde at various time points after the challenge. The fixed lungs were embedded in paraffin, and multiple step sections (taken at 50-μm intervals) were differentially stained with Wright-giemsa for the identification of eosinophils and viewed at $\times$1000. The individual eosinophils were counted from 100 randomly selected high-powered fields per mouse lung at each time point using multiple step sections of lung. To count the eosinophils, strict criteria were followed. The eosinophils counted were in juxtaposition to an airway. This assured the enumeration of only those eosinophils within or immediately adjacent to an airway. The eosinophils were enumerated in lungs in a blinded fashion and analyzed only after all counts were completed in a given experiment. The inflammation observed was associated around the airway with little or no alveolitis.

Collection of Bronchoalveolar Lavage (BAL) Fluid

Lungs from mice were perfused with 1 ml of PBS via intratracheal injection with a 1-ml syringe and 26-gauge needle. After 30 to 40 seconds, the PBS was collected by aspiration with the same syringe and needle. Between 700 to 800 μl could routinely be recollected from the perfused lung. The cells were then collected by centrifugation and resuspended in fresh PBS and cytospun onto a glass slide. The cytospins were then differentially stained with eosin and hematoxylin. The percentage of cells were then determined by counting the number of eosinophils per 200 total cells. The total number of cells from control groups compared with anti-SCF-treated allergic animals did not significantly differ, and thus the percent change in eosinophils reflected a real change in eosinophil numbers within these studies. Histamine levels were measured in the BAL by enzyme-linked immunosorbent assay using commercially available kits (Amac, Inc, Westbrook, MA).

Production of Anti-SCF Antibodies

Rabbit anti-murine SCF antibodies were prepared by multiple-site immunization of New Zealand White rabbits with recombinant murine SCF (Genzyme) in CFA. Polyclonal antibodies were titered by direct enzyme-linked immunosorbent assay and specificity verified by the failure to cross-react to mIL-3, mIL-1α, mTNF, mMIP-1α, IL-6, mJE, mMIP-1β, hMCP-1, hIL-8, hRANTES, hMIP-1α, hTNF, and hMIP-1β. The IgG portion of the serum was purified over a protein A column and used in a sandwich enzyme-linked immunosorbent assay.

In Vivo *Neutralization of SCF*

Neutralization of SCF was carried out using a polyclonal rabbit anti-murine SCF antibody developed in our laboratory as above. The protein A column purified anti-SCF or control antibody was administered intratracheally with SEA at time 0. The BAL fluid was harvested at various time points after SEA challenge and analyzed for leukocyte content. Likewise, paraffin-embedded lung sections were stained, and the peribronchial eosinophil accumulation was quantitated at the various time points after the challenge.

Measurement of Airway Hyperreactivity

Airway hyperreactivity was measured using a Buxco mouse plethysmograph that is specifically designed for

the low tidal volumes (Buxco, Troy, NY), as previously described.[23] Briefly, the mouse to be tested was anesthetized with sodium pentobarbital and intubated via cannulation of the trachea with an 18-gauge metal tube. The mouse was subsequently ventilated with a Harvard pump ventilator (tidal volume, 0.4 ml; frequency, 120 breaths/minutes; positive end-expiratory pressure, 2.5 to 3.0 cm H_2O), and the tail vein was cannulated with a 27-gauge needle for injection of the methacholine challenge. The plethysmograph was sealed and readings monitored by computer. Because the box is a closed system, a change in lung volume was represented by a change in box pressure (P_{box}) that was measured by a differential transducer. The system was calibrated with a syringe that delivered a known volume of 2 ml. A second transducer was used to measure the pressure swings at the opening of the trachea tube (P_{aw}), referenced to the body box (ie, pleural pressure), and to provide a measure of transpulmonary pressure ($P_{tp} = P_{aw} - P_{box}$). The trachea transducer was calibrated at a constant pressure of 20 cm H_2O. Resistance is calculated by the Buxco software by dividing the change in pressure (P_{tp}) by the change in flow (F) ($\delta P_{tp}/\delta F$; units = cm H_2O/ml/sec) at two time points from the volume curve based on a percentage of the inspiratory volume. Once the mouse was hooked up to the box it was ventilated for 5 minutes before acquiring readings. Once baseline levels were stabilized and initial readings were taken, a methacholine challenge was given via the cannulated tail vein. After determining a dose-response curve (0.001 to 0.5 mg), an optimal dose was chosen, 0.1 mg of methacholine. This dose was used throughout the rest of the experiments in this study. After the methacholine challenge, the response was monitored and the peak airway resistance was recorded as a measure of airway hyperreactivity.

Intratracheal Instillation of SCF

Recombinant murine SCF (Genzyme) was instilled directly in the airways of normal CBA/J mice at various concentrations (5 to 500 ng) in 25 μl of saline. Subsequently, mice were assessed for their airway hyperreactivity responses.

Statistics

Statistical significance was determined by analysis of variance, and significance was determined with P values <0.05.

Results

Induction of Allergen-Induced Airway Hyperreativity Can Be Attenuated by Inhibition of SCF

In previous studies in our laboratory, we have demonstrated that neutralization of SCF in the airway significantly reduced histamine levels in the BAL fluid and eosinophil accumulation in and around the airway during an allergic response.[19] In the present studies we were interested in whether the accompanying allergen-induced airway hyperreactivity was also attenuated when we neutralized SCF. Sensitized mice were rechallenged intratracheally with allergen in the presence of either anti-SCF specific antibodies (purified IgG) or control IgG (0.5 mg). We have previously demonstrated that peak airway hyperreactivity occurs between 8 and 24 hours. Therefore we examined airway hyperreactivity at 24 hours after rechallenge.[24] When we examined airway hyperreactivity using an optimal dose of methacholine (100 μg/kg), we found a very significant reduction in airway resistance (Figure 1) in animals given anti-SCF. This reduction in airway resistance was accompanied by a significant reduction in peribronchial eosinophil accumulation (Figure 2), as previously described.[19]

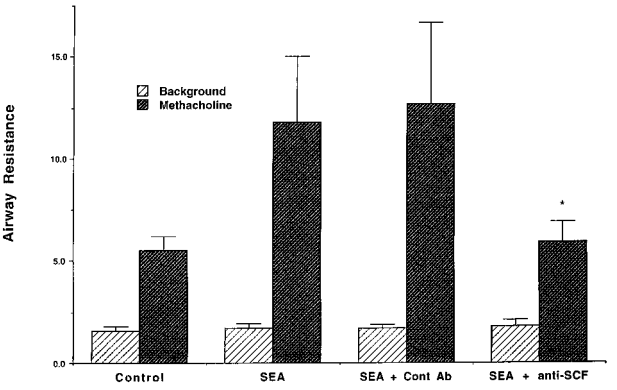

Figure 1. Neutralization of SCF during allergic airway inflammation attenuates airway hyperreactivity. Sensitized animals were rechallenged with allergen containing purified 0.5 mg polyclonal IgG anti-SCF or control antibody via an intratracheal instillation. At 24 hours after allergen, challenge animals were assessed for the presence of airway hyperreactive responses after a challenge of an optimal dose of methacholine (100 μg/kg). Data represent mean ± SE of 8 to 10 animals/group. $^*P < 0.05$

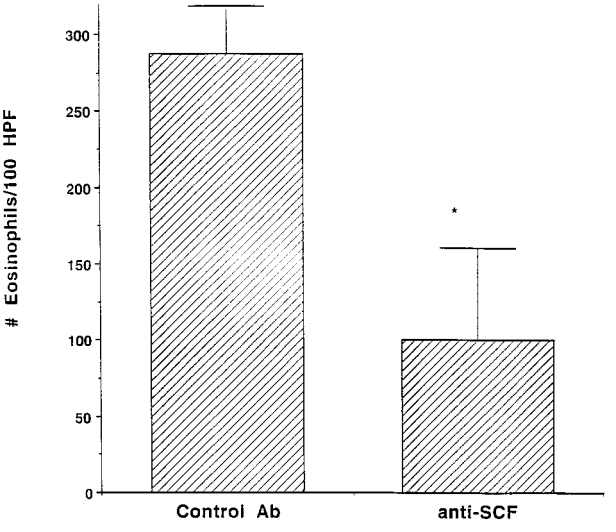

Figure 2. Neutralization of SCF decreases peribronchial eosinophil accumulation during allergic airway responses. Histological sections from the lungs of allergic mice were examined for peribronchial eosinophil accumulation after treatment with anti-SCF or control antibody during an allergic airway response. Eosinophils were enumerated in 100 high-power fields from multiple tissue sections of each mouse. Data represent the mean ± SE from 8 to 10 mice/group. $^*P < 0.05$

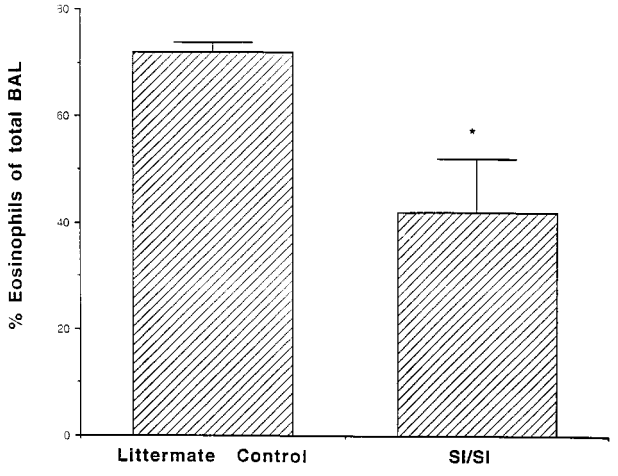

Figure 3. SCF-deficient mice (Sl/Sld) have decreased eosinophil accumulation within their airways. Allergen-sensitized littermate or SCF-deficient mice were challenged intratracheally with allergen, and BAL fluid samples were taken and percent of eosinophils determined via differential staining on cytospin-fixed slides. Data represent mean ± SE of six mice/group. $^*P < 0.05$

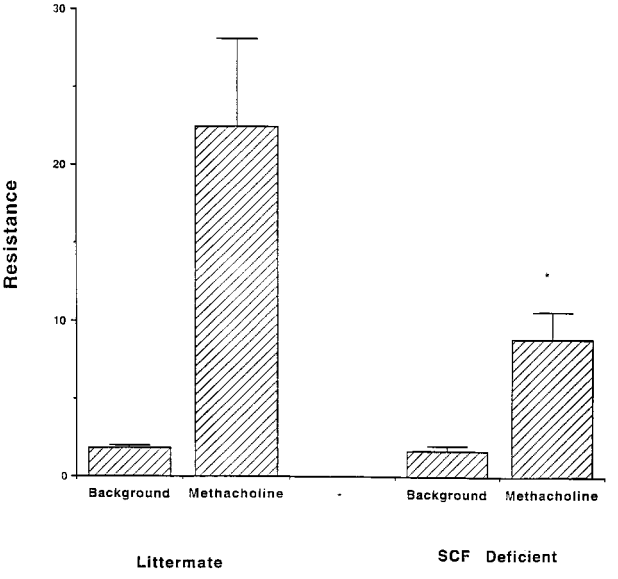

Figure 4. SCF-mutant mice have decreased airway hyperreactivity responses. Allergen-sensitized littermate or SCF-deficient mice were challenged intratracheally with allergen and assessed for airway hyperreactivity responses to an optimal dose of methacholine (100 μg/kg). Data represent change in resistance in six mice/group. $^*P < 0.05$

Reduction in Airway Hyperreactivity and Eosinophil Accumulation in Sld-Mutant Mice

To examine the role of SCF further, we have used SCF-mutant mice (Sld) that have few or no tissue mast cells and compared the allergic responses with their associated littermate controls. When sensitized Sld mice were rechallenged with specific allergen, they demonstrated an attenuated eosinophil accumulation response within the airway at 48 hours after challenge compared with their littermate controls (Figure 3). Because these mice have a mutation (inabilty to make the transmembrane form of SCF) that affects the hematopoesis of a number cell populations, we examined the cytokine profile in allergen rechallenged spleen cells. These studies demonstrated that SCF-deficient mice made similar amounts of a number of cytokines as littermate controls *in vitro* when stimulated with SEA or Con A, including interleukin (IL)-4 (data not shown). Thus, the reduced responses within the airway could not be attributed to a reduction in the sensitization process. When we examined the airway hyperreactivity responses in these mice at 24 hours after allergen challenge, we observed a significant reduction in airway hyperreactivity that resembled the anti-SCF-treated animals in the previous studies (Figure 4). Thus, the SCF-deficient mice have an altered allergic airway eosinophilic and hyperreactive response.

Intratracheal SCF Administration Directly Induces Changes in Airway Hyperreactivity

Previous data have indicated that SCF can induce activation of tracheal smooth muscle cell contraction[21] possibly via mast cell activation. To determine whether SCF can directly induce airway hyperreactivity, we injected normal mice with various doses of recombinant SCF. As shown in Figure 5, the intratracheal injection of SCF induced a dose-dependent increase in airway hyperreactivity at 4 hours postinjection. As little as 5 ng injected intratracheally induced a significant increase in airway resistance, whereas 50 and 500 ng induced a similar level of increased resistance that was significantly higher than that induced by 5 ng/mouse. In addition, we examined the airway hyperreactivity response in a time dependent manner after SCF (50 ng/mouse) instillation and observed that the airway hyperreactivity was not significantly increased at 2 hours, significantly increased at 4 hours, and maintained at 24 hours after SCF instillation (Figure 6). These data suggest that SCF may be playing a direct role in inducing airway hyperreactivity or a role in prolonged mast cell activation correlating with the maintenance of airway hyperreactivity at 24 hours. We also found that a significant increase in histamine in the SCF compared with saline control instilled lungs (15.5 ± 1.7 nM *versus* 8 ± 2.0 in BAL), suggesting a degranulation

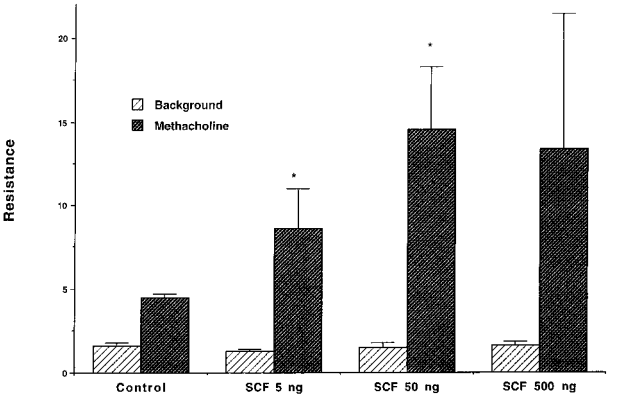

Figure 5. Instillation of SCF induces airway hyperreactivity. Increasing doses of SCF were intratracheally instilled into the lungs of normal, unsensitized mice. After 4 hours the mice were assessed for increased airway hyperreactivity responses. Data represent mean ± SE of four to six mice/group. $^*P < 0.05$.

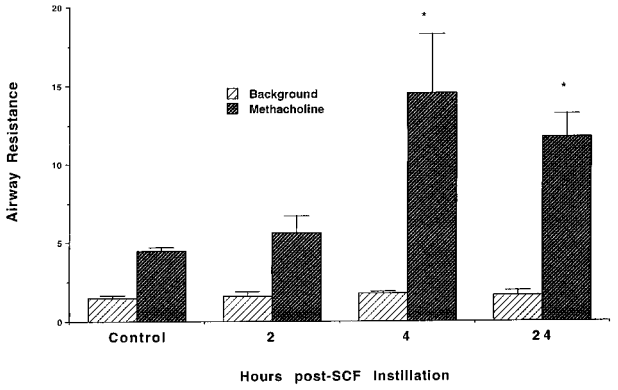

Figure 6. Time course of SCF-induced airway hyperreactivity in normal mice. SCF (50 ng/ml) was intratrachally instilled into normal, unsensitized mice, and airway hyperreactivity was assessed at various time points after treatment. The data represent four to six mice/group. $^*P < 0.05$.

event. To determine whether SCF was acting through the mast cell, we administered SCF (50 ng/mouse) down the airway of mast cell-deficient mice (Sl/Sld), which have a functional c-kit receptor, and compared the airway hyperreactivity responses with littermate controls. In these studies, depicted in Figure 7, the mast cell-deficient mice had a significantly lower increase in airway resistance. In fact, the level of change in the SCF-mutant mice was similar to those observed in vehicle control mice, whereas the littermate controls treated with SCF had a significantly increased airway hyperreactive response. Thus, these results suggest that SCF-induced hyperreactivity was dependent on mast cell activation.

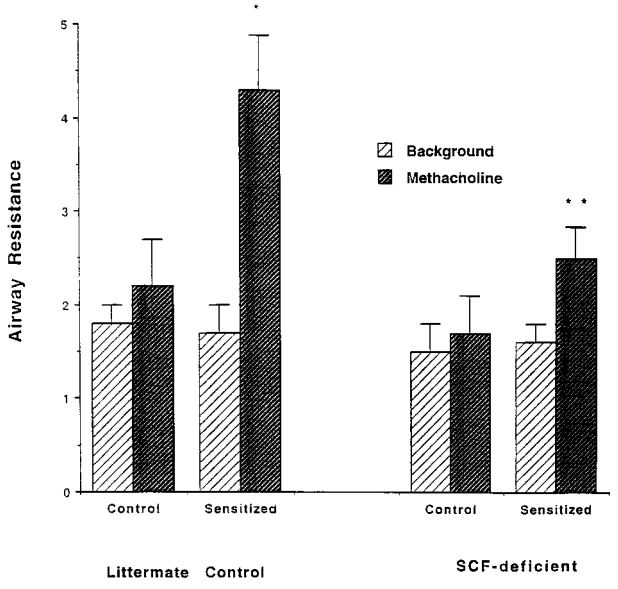

Figure 7. Instillation of SCF into SCF-mutant mice does not induce an airway hyperreactive response. SCF (50 ng/ml) was intratrachally instilled into normal, unsensitized littermate control, and SCF-mutant mice and airway hyperreactivity was assessed at various time points after treatment. Vehicle control mice demonstrated a change in resistance to methacholine of 0.7 ± 0.2, a similar response as the SCF-mutant mice instilled with SCF. The data represent five mice in each group. $^*P < 0.05$

Discussion

The mechanisms involved in the exacerbation of airway reactivity in asthmatic patients are not entirely clear. However, it appears that leukocyte accumulation and activation within and around the airway significantly exacerbates the response.[3–6] In these studies we have examined the role of a mast cell activating and degranulation cytokine, SCF, in the induction of airway hyperreactivity. We have previously demonstrated that SCF plays a significant role in histamine release and eosinophil accumulation during allergic airway responses.[19] The data in the present study further demonstrate that SCF appears to play a significant role in inducing airway hyperreactivity during allergen-specific exacerbations and when SCF was directly injected intratracheally into normal mice. Interestingly, SCF-deficient mice demonstrated a significant decrease in allergen-induced responses, including eosinophil accumulation and changes in airway resistance. These observed decreases in responses were not due to a decrease in allergen-associated lymphocyte deficiencies as the SCF-mutant mice did not demonstrate a decrease in development of the Th2 directed allergen response (IL-4). Because the Sld-mutant mice are deficient in pulmonary mast cells, it is not clear from these studies whether the decreased responses observed in these mice were due to the lack of mast cells or the lack of SCF. However, because recombinant SCF directly induces mast cell degranulation and airway hyperreactivity in normal mice, and this response was significantly attenuated in the SCF-deficient mice, we would suggest that it likely functions via degranulation of local mast cell populations and release of acute mediators.

The role of SCF in these studies appears to operate via mast cell activation that can directly mediate changes in airway physiology or indirectly affect airway physiology through the initiation of eosinophil accumulation. In both cases the mast cell appears to play a pivotal role in the responses. In addition, SCF enhances eosinophil adhesion events via VLA-4/VCAM-1 interactions.[17] This latter function of SCF biology in eosinophil accumulation may play an important role in the late phase of asthmatic reactivity in the lung. The role of mast cells for the induction of airway hyperreactivity in animal models of allergic airway responses is controversial. Studies using the c-kit-deficient mice (WW^v mutation) have had conflicting reports in allergic models,[31–33] however, our studies with mast cell-deficient mice and direct activation of mast cells with intratracheal SCF administration both point to the conclusion that mast cells contribute to the exacerbation of airway hyperreactivity. Furthermore, SCF and mast cells may play a direct role in airway hyperreactivity, whether via released mediators or induction of eosinophil accumulation. We have recently described that fibroblast-expressed SCF can specifically drive eotaxin production from mast cells *in vitro*,[34] possibly explaining the relationship between SCF, mast cells, and eosinophils.

The role of SCF in the allergic response appears to have multiple components. SCF can serve as a mast cell degranulating and activating factor that augments the IgE mediated events.[11,12] SCF may initially induce the

release of mediators that can play a role in the airway hyperreactivity responses such as leukotrienes that are elevated after SCF injection into normal mice (unpublished data). The cysteinyl leukotrienes have been previously identified as long lasting inducers of airway hyperreactivity,[35,36] and their release into the airway may help explain the maintenance of the response at 24 hours. Previous studies with SCF have demonstrated that it can specifically activate mast cells, induce IL-6 production, alter the arachadonic acid production profile from mast cells, and initiate prostaglandin production.[12,37,38] Alternatively, SCF may have the ability to directly activate other important cell types within the airway, such as smooth muscle cells that would control the airway contraction. However, the data with SCF down the airway of mast cell-deficient mice that have a functional SCF receptor suggests that the mast cell may be the primary cell population that is involved in the response. Because SCF production from alveolar macrophages can be induced by tumor necrosis factor,[19] and SCF can directly induce airway hyperreactivity, SCF may play a significant role in nonallergen-induced asthmatic exacerbations of airway reactivity such as in viral infections. This may be an important issue to elucidate for targeting therapeutic intervention.

The ability of a single cytokine, SCF, to not only augment but to directly mediate inflammatory and hyperreactive responses *in vivo*, may have global implications in allergic, as well as nonallergic diseases. Defining the role of SCF in allergic airway responses will help to delineate a mechanistic approach to altering mast cell-dependent responses and aid in implementing therapeutic modalities to alleviating the activation of airway injury and hyperreactivity.

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