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# Stem cell factor and IgE-stimulated murine mast cells produce chemokines (CCL2, CCL17, CCL22) and express chemokine receptors

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**Abstract.** *Objective and design:* In the present study we investigated the effect of SCF and/or IgE on histamine, TNF-$\alpha$ and chemokines released from bone marrow-derived mast cells (BMMC) as well as chemokine receptor expression.
*Material and methods:* BMMC were derived from femoral bone marrow of CBA/J mice. The purity of BMMC was >98% after 3 weeks. BMMC ($2.5 \times 10^6$ cells/well) were incubated in the presence or absence of either SCF, IgE plus DNP or a combination of SCF and IgE for 6 and 18 h. Cell-free supernatants were recovered to measure CC chemokines, TNF-$\alpha$ and histamine release utilizing ELISA assays. CC chemokine family receptors were detected by RT-PCR analysis, and confirmed using functional chemotactic assays.
*Results:* Histamine levels were comparable between SCF and IgE stimulated cells, whereas TNF-$\alpha$ production was significantly greater after IgE compared to SCF stimulation. SCF and/or IgE-stimulated BMMC released CC chemokines, CCL22 (MDC), CCL17 (TARC) and CCL2 (MCP-1). Increased mRNA expression of CCR1, CCR2, CCR3, and CCR5 was detected in SCF and IgE-stimulated BMMCs. Functional chemotactic assays confirmed the expression data.
*Conclusion:* SCF and IgE can up-regulate the expression of chemokines and chemokine receptors on mast cells. Thus, SCF may play a significant role in their activation and inflammation during allergic responses.

**Key words:** SCF – Mast cells – CC chemokine – CC chemokine receptor – Histamine – TNF-$\alpha$

## Introduction

Mast cells are multifunctional cells that can initiate or modulate various inflammatory processes. These cells contain many preformed mediators that, when released, may induce initial and necessary interactions between circulating leukocytes and the endothelium of postcapillary venules [1]. The activation and degranulation of mast cell populations are responses that can be mediated by either antigen-specific, surface bound IgE or by stem cell factor (SCF) [2]. IgE-mediated mast cell activation induces immediate mast cell degranulation that constitutes the primary mechanism that drives the allergic responses in terms of type I hypersensitivity response. During this activation, mast cells release preformed and newly synthesized pro-inflammatory mediators including histamine, heparin, proteases, prostaglandin $D_2$, leukotriene $C_4$, and cytokines. In addition, murine mast cells challenged in an IgE-dependent manner or with other stimuli can produce multiple chemokines, including CCL3 (MIP-1$\alpha$), CCL4 (MIP-1$\beta$) and CCL2 (MCP-1) that can initiate and perpetuate an inflammatory cascade [3].

CC chemokines are chemotactic cytokines that can induce mediator secretion and chemotaxis of leukocytes [4]. The secretion of chemokines has been detected in a wide variety of cells, including macrophages, endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, lymphocytes, neutrophils, eosinophils and mast cells [5, 6]. High levels of chemokines have been detected during in vivo inflammatory reactions, such as bacterial sepsis [7], autoimmune reaction [8], and bronchial asthma allergic disorders [9–11]. However, the specific mast cell-derived chemokines that function in these different inflammatory processes have not been completely elucidated.

Stem Cell Factor (SCF) or c-kit receptor ligand has been identified as a primary cytokine involved in mast cell differentiation and activation [12–15]. SCF binds to its surface receptor, c-kit, which is a member of the receptor tyrosine kinase family and SCF has a role in mast cell survival, as mast cells cultured without SCF undergo apoptosis [16]. Furthermore, SCF enhances IgE-dependent human mast cell mediator release, including the generation and release of cytokines [17–19]. Thus, the prolonged activation of mast cell populations by SCF, after initial IgE-mediated events or by itself, may play a significant role in late phase asthma responses.

In the present study we investigated the role of both SCF and IgE-dependent mechanisms for histamine release and

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chemokine and TNF-$\alpha$ production by bone marrow-derived murine mast cells. In addition, we evaluated the ability of SCF and/or IgE-mediated mast cell activities to increase the expression of specific chemokine receptors. Our data demonstrated that SCF is a potent mast cell activator, which can stimulate the expression of both CC chemokine and chemokine receptors, TNF-$\alpha$ and induce the release a mast cell mediator histamine.

## Materials and methods

### *Isolation and expansion of bone marrow-derived mast cells*

Primary mast cell lines were derived from femoral bone marrow of pathogen-free CBA/J mice (Jackson Laboratory, Bar Harbor, ME) [20]. The cells were incubated with Dulbecco's modified Eagle's medium (DMEM – Bio Whittaker, Walkersville, MA) supplemented with 1 mM L-glutamine, 10 mM N-2-hydroxiethylpiperazine-NI-2-ethanesulfonic acid (HEPES), antibiotics, and 15% fetal calf serum (FCS) combine with 10% T-stimulated rat splenocyte culture supplement medium with IL-3 (15 ng/ml) and SCF (15 ng/ml). Without addition of exogenous SCF there was poor mast cells growth. The media was changed every three days. By the end of 2–3 weeks, a nonadherent population of large granular cells was observed. These isolated cells appeared homogeneous in cytospin preparations stained by Diff Quik (Baxter, MacGraw Park, IL) with typical mast cell granular appearance. The homogeneity of these cell lines was determined by flow cytometric analysis of surface markers, by histamine release assays, and by electron microscopy. In particular, these cells were *c-kit* positive (SCF receptor) but were negative for CD3, CD4, CD8, CD23, B220, and F480 by flow cytometric. The purity of BMMC was >98%. These cell lines were routinely expanded, as described above, for 3 to 6 weeks. Before each experiment, BMMC were washed and new medium was added without SCF.

### *Stimulation of BMMC with murine recombinant SCF and/or IgE plus dinitrophenyl (DNP)*

Bone marrow-derived mast cells ($2.5 \times 10^6$ cells/well), were incubated in complete DMEM with 15% FCS in the presence or absence of either SCF in different concentrations (0.1, 1, 10, 100 and 200 ng/ml), IgE (2 g/ml) plus DNP (100 ng/ml) or a combination of SCF (100 ng/ml) and IgE plus DNP at 37 °C in 5% $CO_2$ for 6 and 18 h. After stimulation, cells were centrifuged and the cell-free supernatant recovered to measure CC chemokines, TNF-$\alpha$ and histamine release.

### *Quantification of CC chemokines and TNF-$\alpha$*

Extracellular immunoreactive murine CC chemokines (MCP-1, MDC, TARC) and TNF-$\alpha$ were quantified using a modified double ligand procedure of enzyme-linked immunosorbent assay (ELISA) [21]. This ELISA method consistently detected CC chemokine or TNF-$\alpha$ levels over 20 pg and did not cross-react with other cytokines. 96-well-flat-bottomed microtiter plates were coated with 50 µl/well of either rabbit anti-CC chemokine antibodies or anti-TNF-$\alpha$ (1 g/ml in 0.6 M NaCl, 0.26 M $H_3BO_4$, and 0.08 N NaOH, pH 9.6) for 16 h at 4 °C, and the washed (PBS, pH 7.5, 0.05% Tween 20). Blocking of nonspecific binding sites was accomplished by incubating plates with PBS containing 2% BSA for 90 min at 37 °C. Plates were rinsed thoroughly with wash buffer and aqueous samples were added. Following a 1-hours incubation at 37 °C, plates were washed and biotinylated rabbit either anti-CC chemokine or anti-TNF-$\alpha$ Ab was added and incubated for 30 min at 37 °C. Plates were then washed and chromogen substrate added, and they were subsequently read at 490 nm.

### *Histamine release assays.*

Histamine ELISA kits (Immunotech, Westbrook, ME) were used to determined histamine levels in supernatants from stimulated mast cells. As positive controls, mast cells were exposed to compound 48/80 (Sigma), a mast cell degranulator, and cells were sonicated to determine total intracellular histamine levels.

### *Reverse transcription (RT)-PCR analysis*

Total cellular RNA was extracted from $2{,}5 \times 10^6$ BMMC using TRIzol reagent (Life Techonologies). Synthesis of first-strand cDNA was performed in 20 µl of reaction mixture containing 2 µg RNA, 1 µl dNTP 100 mM, 5 µl oligo $(dT)_{12-18}$ primer 0.5mg/ml, 5 µl Rnase inhibitor 40 U/ml, 5 µl reverse transcriptase 25 U/l (Boehringer Mannheim, Mannheim, Germany) incubated at 650 C for 7 min, and then incubated at 37 °C for 1 h. The AMV reverse transcriptase was denatured by 90 °C for 5 min and then placed on ice. Sequences of the primers for the amplification were CCR1 (sense 5′-gaccagcatctacctgttca-3′; and antisense, 5″-gcagaaacaaatacactcag-3′), CCR2 (sense 5′-cacgaagtatccaagagc-3′; and antisense 5′-catgctcttcagctttttac-3′), CCR3 (sense 5′-tgggcaacatgatggttgtg-3′; and antisense 5′-gctgtcttgagactcatgga-3′), CCR4 (sense 5′-cctgcctcctctctactcct-3′; and antisense 5′- acgtgtggttgtgctctgtg-3′), CCR5 (sense 5′-gctgaagagcgtgactgata-3′; and antisense 5′-gaggactgcatgtataatga-3′). Reverse transcriptase reaction mixture was used in the polymerase chain reaction (PCR) in 20 µl final volume, 0.5 µl of each dNTP 100 mM, 2 µl of each primer 300 ng/l and 1.5 µl of Taq DNA polymerase 5 U/ml (Boehringer Mannheim). The mixture was incubated in a thermocycler using the following temperature profile: denaturation step at 94 °C for 4 minutes, followed by 35 cycles (CCR1, CCR4 and CCR5) and 30 cycles (CCR2 and CCR3) of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 45 seconds, and extension at 72 °C for 45 seconds. The final extension step was 72 °C for 10 min. PCR samples were run on a 2% agarose gel stained with 10-mg/ml ethidium bromide, and the PCR products were visualized with UV light and photographed.

### *Mast cell chemotaxis*

Mast cell migration was quantitated by a modification of a Boyden chamber technique described previously [29]. Mast cells were suspended at $3 \times 10^6$ cells/ml in DPBS plus 0.5% BSA and were placed in the top wells of the microchemotaxis chamber. Bottom wells were filled with CC chemokines in the final concentration of 50 ng/ml or assay medium as negative control. An 8-m-pore-size polycarbonate filter separated the upper wells containing the cells from the control and chemokines samples in the bottom wells. The polycarbonate filters used in these experiments were coated with fibronectin (10 g/ml) at room temperature overnight and were then air-dried. The chambers were incubated for 2 h at 37 °C in a 5% $CO_2$ moist atmosphere, and the filters were then carefully scraped of nonmigrating cells, fixed with methanol and stained with Diff-Quik. Mast cell migration was quantitated by counting the number of mast cells migrating completely through the matrix-coated filter in 10 high-powered fields (hpf) in triplicate samples. The data are expressed as the average number of countable adherent cells per hpf (± SEM).

### *Statistical analysis.*

Statistical significance was determined by ANOVA, and significance was determined with p values $< 0.05$.

**Table 1.** Release of TNF-$\alpha$ and histamine from bone-marrow murine mast cell stimulated with SCF, IgE or a combination of IgE plus SCF.

| Stimulus | TNF-$\alpha$ (ng/ml) | | | Histamine (nM) | | |
|---|---|---|---|---|---|---|
| | 1 h | 6 h | 18 h | 1 h | 6 h | 18 h |
| Control | < 0.1 | < 0.1 | < 0.1 | 97.20 ± 0.4 | 138.9 ± 8.4 | 118 ± 10.3 |
| SCF | 0.05 ± 0.001 | 0.38 ± 0.08* | 0.26 ± 0.1* | 491 ± 71.5* | 626 ± 26.7* | 262 ± 24.4# |
| IgE | 2.65 ± 0.1 | 4.11 ± 2.1* | 5.80 ± 0.1* | 753 ± 15.5* | 355.2 ± 2.8* | 884 ± 37.1* |
| IgE + SCF | 3.30 ± 0.1 | 14.53 ± 1.3** | 10.39 ± 0.2** | 1398.9 ± 173.6** | 1475.8 ± 154.3** | 1589 ± 156.3** |

BMMC were stimulated with SCF (200 ng/ml), IgE (2 µg/ml) plus DNP (100 ng/ml) or a combination of SCF, IgE plus DNP for 6 h or 18 h at 37 °C in DMEM. After incubation, cells were centrifuged and the cell-free supernatants recovered. TNF-$\alpha$ and histamine levels were measured by ELISA. The results represent the mean SEM of duplicate culture from one typical experiment. Similar data were obtained in two other experiments. *$p < 0.0001$, when compared with control. **$p < 0.01$, when compared with IgE group. #$p < 0.005$, when compared with control.

## Results

### *Detection of* TNF-$\alpha$ *and histamine in the SCF and/or IgE-stimulated BMMC*

Mast cells are important cellular sources of different multifunctional cytokines [23] and have been identified as the only resident cell capable of storing TNF-$\alpha$ in cytoplasmic granules. Several studies demonstrate that mast cell-derived TNF-$\alpha$ serve as a central component of host defense against bacterial infection and is crucial for establishing cytokine networks that lead to the recruitment of leukocytes [24, 25]. Our initial investigation demonstrated that although SCF (200 ng/ml) was able to induce release of mast cell-derived TNF-$\alpha$, a significantly higher release of TNF-$\alpha$ was observed in mast cell culture supernatant after IgE stimulation (Table I). As previously reported, SCF (200 ng/ml) was able to further up-regulate TNF-$\alpha$ production at both 6 and 18 h post IgE stimulation (Table I). These results suggest that mast cells have differential TNF-$\alpha$ production dependent on the stimulus and that both IgE and SCF induce de novo production of TNF in addition to that released immediately upon stimulation.

Histamine release by mast cells has an important function during an immediate reaction, as it can induce vascular permeability causing vasodilatation and edema [26]. In mast cell cultures increased histamine levels were present by 1 h after SCF stimulation and remained elevated for the next 6 h. After 18 h, histamine levels in the cell culture were diminished. During IgE stimulation, histamine levels were also present by 1 h; continued to increase at 6 h and by 18 h even higher levels of histamine could be observed. SCF (200 ng/ml) had a synergistic effect on histamine release when combined with IgE stimulation (Table I). These results indicate that SCF has a similar propensity as IgE to induce release of histamine.

### *Production of CC chemokines by bone marrow-derived mast cells (BMMC)*

In order to investigate the role of SCF and/or IgE stimulation for chemokine production by murine mast cells (BMMC), SCF, IgE, or a combination of SCF and IgE were used. We observed that SCF (200 ng/ml)-stimulated BMMC release MDC, TARC and MCP-1, (Fig. 1) into the culture supernatants. Lower doses of SCF (0.1–100 ng/ml) showed no significant differences when compared to control (data not shown). The levels of MCP-1 were further augmented at 6 h when IgE and SCF (100 ng/ml) were added concomitantly (Figure 1E). Interestingly, both SCF and IgE induced CCL22 (MDC) production at very high levels, while TARC production was much more modest. However, the level of CCL17 (TARC) was increase ~4-fold when IgE and SCF (100 ng/ml) were added concomitantly (Fig. 1D). The levels of MDC subsequently increase ~5-fold when both SCF and IgE were added together to the cultured BMMC (Fig. 1B). Overall, these data suggest that mast cells stimulated by SCF and/or IgE are an important source of CC chemokine production, CCL22 (MDC), CCL17 (TARC) and CCL2 (MCP-1).

### *SCF and IgE-induced CC chemokine receptor expression and BMMC chemotaxis*

Previous data indicated that chemokines have a role in mast cell activation and degranulation. To determine whether SCF and/or IgE could induce the up-regulation of CC chemokine receptors in BMMC, RT-PCR analysis was used. As shown in Fig. 2 both SCF and IgE stimulation were able to signifi-

**Table 2.** Mast cell chemotaxis activity in absence of SCF.

| Stimulus | Mast cells/10 HPF Absence of SCF |
|---|---|
| Control | 7.5 ± 5.5 |
| MIP-1$\alpha$ | 232.5 ± 12.5* |
| MCP-1 | 367 ± 31* |
| Eotaxin | 171 ± 69* |
| MDC | 15 ± 5 |
| MIP-1$\beta$ | 198 ± 18.5* |

BMMC were obtained from primary bone marrow cultured for 3 weeks in DMEM supplemented with 15% FCS, IL-3 and SCF as described in Materials and Methods. After 3 weeks, cells were washed and suspended in DMEM in absence of SCF. Cells were loaded in the upper chamber and chemotaxis to CC chemokines (50 ng/ml) were observed after 2 h at 37 °C. The results represent the mean SEM of duplicate culture from one typical experiment. Similar data were obtained in two other experiments. *$p < 0.001$ when compared with control.

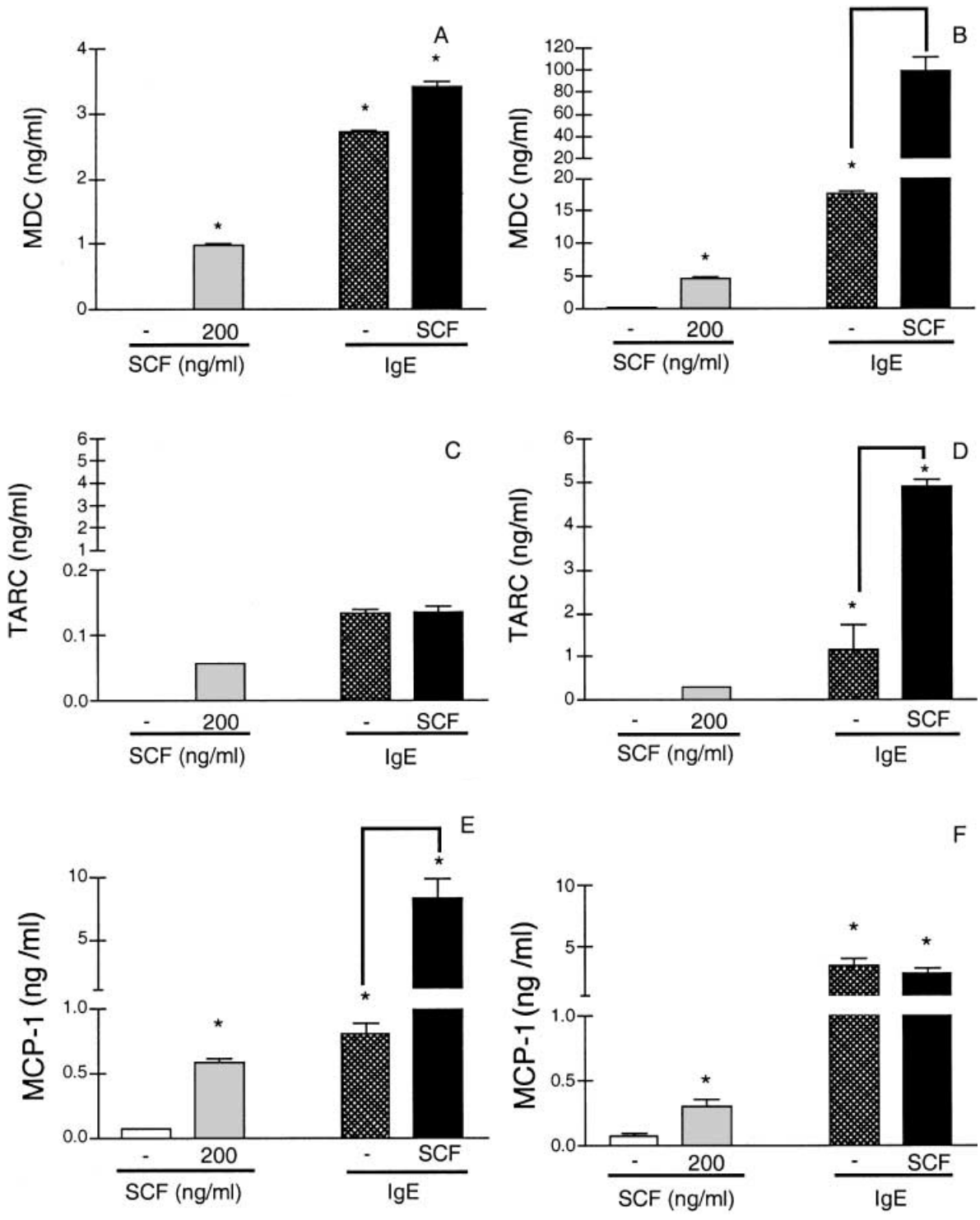


**Fig. 1.** Incubation of normal mast cells with SCF (200 ng/ml) and/or IgE (2 μg/ml) + DNP induces MDC (A, B), TARC (C, D), MCP-1 (E, F) production by 6 h and 18 h respectively. The results represent the means ± SEM of triplicate cultures from one typical experiment. Similar data were obtained in two other experiments. *$p < 0.001$. No significant increase in the chemokines was observed at 1 h time points.

cantly up-regulate CCR1, CCR2, CCR3 and CCR5 mRNA expression in BMMC, while co-activation with both stimuli did not further upregulate the receptors.

In order to ascertain whether these receptors were functionally expressed on mast cells we utilized mast cell chemotaxis assays. The data in Table II indicates that mast cells grown in the presence of SCF have the ability to migrate to CC chemokine ligands that are specific for the various chemokine receptors. The migration patterns match the RT-PCR expression patterns described in Fig. 3. That is that ligands for CCR1 (MIP-1$\alpha$), CCR2 (MCP-1), CCR3 (eotaxin), and CCR5 (MIP-1$\beta$), but not MDC and TARC (CCR4),

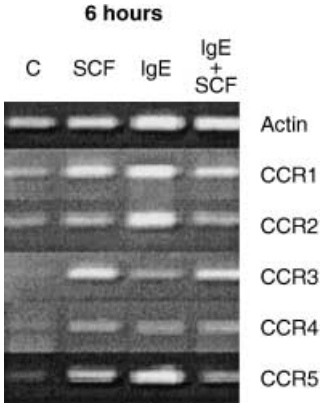


**Fig. 2.** Incubation of bone marrow-derived mast cells with SCF (200 ng/ml), IgE (2 µg/ml) + DNP, or a combination of SCF (100 ng/ml) plus IgE up-regulate CCR1, CCR2, CCR3 and CCR5 expression by 6 h. 2 µg of total mRNA purified from bone marrow-derived mast cell was used in RT-PCR analysis. Similar results were obtained from 2 repeat experiments.

induced the migration of BMMCs in chemotactic assays. No further increase in chemotaxis could be detected when the BMMC were stimulated overnight with SCF or IgE (data not shown). *In additional studies, we also determined that neither MDC (CCL22) nor TARC (CCL17) had any effect on BMMC degranulation (data not shown).*

## Discussion

The participation of SCF in allergic inflammation can induce airway hyperreactivity via direct mast cell activation as well as having a role in eosinophil accumulation [27, 28]. Previous work has demonstrated that SCF and IgE were able to stimulate BMMC to release TNF-$\alpha$, an important pro-inflammatory mediator involved in the allergic inflammatory response [29]. In the present studies, although SCF was able to produce similar levels of histamine to be released from long-term cultures of mast cells, IgE stimulation induced a ten-fold greater increase in TNF. There is evidence for increased TNF-$\alpha$ expression in asthmatic airway [30] and after IgE-mediated activation in sensitized lungs [29, 31]. TNF-$\alpha$ may have an important amplifying effect in allergic asthmatic inflammation [22, 32], with functions that occur in combination with other cytokines as part of cytokine networks [33]. The fact that SCF and IgE stimulation in combination induce 2–3 fold more TNF demonstrates how these activating stimuli synergize during allergic responses. Subsequently, TNF-$\alpha$ would further contribute to the inflammatory response by stimulating airway epithelial cells to produce cytokines and chemokines, including RANTES (CCL5), IL-8 (CXCL8) and GM-CSF [34–36]. This is an issue that may need to be revisited given the described role of SCF for TNF production during bacterial septic response [37]. Interestingly, histamine release was similar between SCF and IgE stimulation.

IgE mediated chemokine release was potentiated by SCF in the same manner as histamine and TNF. Other authors [38, 39] have observed a relationship between histamine and CC chemokines. Treatment of mice with eotaxin (CCL11) [40] produced a time-dependent accumulation of eosinophils that was significantly reduced if the animals were pre-treated with histamine H1 antagonist. Thus, the effect of mediators from mast cells on other cell populations has not been fully appreciated. The fact that SCF also appears to be an important mast cell activator to induce CC chemokine production along with histamine and TNF suggests a generalized upregulation of inflammatory recruitment responses by activated mast cells. The coordination and intensity of the inflammatory response may, in part, be induced by the mast cells during allergic responses. This latter hypothesis is supported by recent investigations of SCF-deficient allergic mice that demonstrated significantly less inflammation and airway hyperreactivity upon allergen challenge [27]. The data in the present studies are consistent with previous investigations demonstrating the ability of SCF to augment MCP-1 production as well as studies demonstrating chemokine production from mast cells during allergic responses [42–48]. SCF up regulating IgE-induced MCP-1 (CCL2) production by stimulated mast cells may be an important mechanism mediating MCP-1 production in allergic inflammatory response. However, the most highly activated chemokine during the mast cell treatment was monocyte-derived chemokine (MDC) (CCL22). MDC was significantly upregulated by both SCF and IgE and was further enhanced five fold higher when mast cells were activated with both SCF and IgE. MDC (CCL22) may have an important role during allergic responses via its ability to induce dendritic cell movement and preferentially recruit Th2 type cells via CCR4 [49]. A recent study has indicated that MDC plays an important role in inflammation and airway hyperreactivity in mouse models of allergic asthma [50]. Thus, this chemokine may be a key mediator produced by stimulated mast cells during allergic responses and contribute to the maintenance of the late phase via the perpetuation of the Th2 type responses. TARC (CCL17) was significantly produced by IgE but not SCF alone; however; it was further upregulated when mast cells are activated with both SCF and IgE. Previous studies have shown TARC (CCL17) production by murine keratinocytic cell line and during the atopic dermatitis-like lesions, a characteristic mast cell degranulated lesions that have high level of IgE [51]. Thus, TARC may contribute to maintenance of the inflammatory process mediated by mast cells and IgE in presence of SCF.

The up regulation of chemokine receptors by SCF and IgE on the BMMCs indicate that during allergic responses the mast cells become more susceptible to chemokines stimulation. This is a significant issue. One aspect of chemokine biology is the ability of several chemokines to cause mast cell activation [52, 53]. In fact, recent studies indicate that activation of mast cells with MCP-1 (via CCR2) can induce mediator release, including histamine and leukotriene release, further exacerbating airway hyperreactivity [54]. Although the functional assay that was performed in the present studies was mast cell chemotaxis, it may be the case that a primary role for chemokines in mast cell biology is activation not migration. This may especially be the issue in fixed tissue mast cells in the upper airways where movement is not necessary. The overall impact of multiple chemokine receptors on cells is presently unclear [55], but future investiga-

tions will likely demonstrate a diverse array of chemokine-mediated functions.

Overall, these studies indicate that SCF can upregulate the expression of chemokines and chemokine receptors on mast cells and along with IgE, SCF may play a significant role in the activation and inflammation during allergic responses. Future studies will examine the significance of expressing multiple chemokine receptors on the mast cells and whether there are differences in function when the different receptors are ligated.

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