Stem cell factor-mediated activation pathways promote murine eosinophil CCL6 production and survival

Vladislav Dolgachev,[1] Molly Thomas, Aaron Berlin, and Nicholas W. Lukacs

Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan, USA

Abstract: Eosinophil activation during allergic diseases has a detrimental role in the generation of pathophysiologic responses. Stem cell factor (SCF) has recently shown an inflammatory, gene-activating role on eosinophils and contributes to the generation of pathophysiologic changes in the airways during allergic responses. The data in the present study outline the signal transduction events that are induced by SCF in eosinophils and further demonstrate that MEK-mediated signaling pathways are crucial for SCF-induced CCL6 chemokine activation and eosinophil survival. SCF-mediated eosinophil activation was demonstrated to include PI-3K activation as well as MEK/MAPK phosphorylation pathways. Subsequent analysis of CCL6 gene activation and production induced by SCF in the presence or absence of rather specific inhibitors for certain pathways demonstrated that the MEK/MAPK pathway but not the PI-3K pathway was crucial for the SCF-induced CCL6 gene activation. These same signaling pathways were shown to initiate antiapoptotic events and promote eosinophil survival, including up-regulation of BCL2 and BCL3. Altogether, SCF appears to be a potent eosinophil activation and survival factor. *J. Leukoc. Biol.* 81: 1111–1119; 2007.

Key Words: *SCF · cell signaling*

INTRODUCTION

Activated eosinophils in allergic disease contribute to the progression of inflammation by producing cytokines and lipid mediators leading to tissue damage by release of toxic proteins and reactive oxygen species [1, 2]. Evidence suggests that eosinophils are associated with development of lung dysfunction and the consequent immunopathology in allergic diseases [3–6]. The ability to promote eosinophil activation for release of inflammatory mediators has been a focus for a number of years and has helped to define how eosinophils might participate in chronic inflammatory diseases. Although a number of factors have been demonstrated to promote eosinophil survival, including IL-3, IL-5, and GM-CSF, there are likely other mediators that also participate in long-term survival in tissues altered by chronic disease [7–10]. In particular, studies have suggested recently that stem cell factors (SCF) may play a role in eosinophil activation during chronic disease [11].

The role of SCF on eosinophil biology has been identified. Although it has been established that SCF has a function in eosinophil maturation within the bone marrow, its role in peripheral tissue has not been elucidated thoroughly. Evidence has identified that SCF up-regulates VLA-4 on eosinophils and augments adherence events to fibronectin and VCAM-1 [12]. Previous data from our laboratory have demonstrated that SCF can increase eosinophil activation and degranulation significantly. This includes the release of eosinophil peroxidase, generation and release of leukotriene C4, and production of chemokines [13]. Blocking of c-kit signaling with anti-SCF antibodies or c-kit intrinsic tyrosine kinase inhibitor (imatinib) reduces lung tissue inflammation, eosinophilia, and mucus production significantly [14–16]. In addition, other studies have identified that substantial levels of SCF can be found in chronic diseases, including fibrotic diseases and asthma, where eosinophils have been implicated in the pathogenesis and severity of the responses [17–20].

The signal transduction events involved in SCF-induced c-kit activation have been investigated in the context of progenitor cell and mast cell maturation/activation pathways [21]. c-Kit is a receptor tyrosine kinase, which constitutes a Type III subfamily that includes platelet-derived growth factor, CSF-1, and fetal liver tyrosine kinase 3 ligand. Upon SCF stimulation, c-kit dimerizes and activates its intrinsic tyrosine kinase and autophosphorylates. This allows the initiation of specific signal transduction pathways [22, 23]. Downstream of c-kit, multiple signal transduction components are activated, including PI-3K, Src family members, the JAK/STAT pathway, and the Ras-Raf-MAPK cascade [22]. Activation of the PI-3K pathway involves association of the Src homology 2 (SH2) domain of the regulatory subunit with phosphorylated receptors for the recruitment of the p85 subunit of PI-3K. Downstream targets for 3′-phospholipids generated by PI-3K include serine/threonine kinases Akt and protein kinase C. PI-3K has been implicated in many aspects of cellular signaling, including DNA synthesis, cell survival, membrane ruffling, chemotaxis, as well as receptor and vascular trafficking [22, 24, 25].

Another aspect of SCF-induced signaling is the activation of the Ras-Raf-MAPK cascade. This pathway is induced by phos-

phorylated tyrosine residues on the SCF-stimulated c-kit receptor, which recruits SH2-containing proteins to the receptor complex that couples the c-kit-associated receptor tyrosine kinase to Ras [22]. The SH2-containing proteins include Grb2, Shc, SH2-containing tyrosine phosphatase 2, and Grap. Subsequently, Grb2-Sos colocalizes with SH2-containing proteins and increases in Ras activity. The next activation step is when Ras interacts with c-Raf-1, which activates MEK, a MAPK [22, 23, 26, 27]. MEK phosphorylates p38 MAPK and ERK1/2 and leads to activation of the downstream component S6 kinase pp90^{rsk}. The MAPK activation pathways have been shown to induce a number of inflammatory and immune mediators via NF-κB activation [28]. None of these pathways has been examined in eosinophils, but as our data demonstrate, they are differentially involved in eosinophil activation and survival.

MATERIALS AND METHODS

Animals

Dr. Fred Lewis at the Biomedical Research Laboratory (Rockville, MD, USA) supplied Swiss Webster mice heavily infected with the *Schistosoma mansoni* helminth parasite. These mice displayed severe infection and significant eosinophilia, and >50% of circulating granulocytes were eosinophils. They were used throughout our studies for elicitation and isolation of eosinophils.

Antigen-elicited peritoneal eosinophil purification

Eosinophils were elicited by injection of thioglycolate plus soluble egg antigen (SEA) into the peritoneum of *S. mansoni*-infected mice. SEA was prepared in our laboratory by grinding isolated eggs from heavily infected *S. mansoni* mice as described previously [29]. The injection of SEA into infected mice induces a pool of circulating eosinophils recruited into the peritoneum in an antigen-specific manner. Using these mice, we could elicit eosinophils that were from an antigen-induced, Th2-associated environment. After 48 h, the mice were peritoneal-lavaged, and the cells were collected. The initial population, which was isolated from the peritoneum, was ~50% eosinophils, and only 2–5% were neutrophils, and 35–45% were mononuclear cells (lymphocytes and macrophages). Adherent cell populations were removed from the population by plastic adherence in tissue-culture dishes for 1 h. The nonadherent cells were washed and resuspended in PBS/BSA (90 μl PBS/BSA per 10^7 cells), and eosinophils were purified by negative selection using immunomagnetic bead-coupled antibodies to exclude contaminating immune cells using the MACS system. The antibodies used were anti-Thy1 (for T cells), anti-B220 (for B cells), and anti-Class II (for APCs). After the plate adherence and MACS separation, the population of cells contained >97% eosinophils contaminated with neutrophils (~1%) and mononuclear cells (1–2%).

Culture of purified eosinophils

Freshly isolated murine eosinophils (3×10^6 per well) were cultivated overnight in RPMI 1640 (Invitrogen, Carlsbad, CA, USA), supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (BioWhittaker, Walkersville, MD, USA), and 10% heat-inactivated FBS (Atlas, San Diego, CA, USA) with IL-5, 5 ng/ml, for survival. Our initial studies demonstrated that freshly isolated eosinophils were highly activated. Therefore, we used the overnight culture in the presence of IL-5 to synchronize the eosinophils, which allowed a starting population of >95% viable cells after overnight culture.

Western blot

Similar protocols to the ones described here were reported elsewhere [30, 31]. Cells were lysed in 1% Nonidet P-40 buffer containing complete protease inhibitor cocktail (Roche, Nutley, NJ, USA) for 30 min on ice with frequent vortexing and centrifuged (1200 g; 5 min at 4°C). Equal amounts of protein from a detergent-soluble fraction were separated by 12.5% SDS–PAGE, electrotransferred onto polyvinylidene difluoride membranes, and probed with

phospho-Akt (Ser473), phospho-MEK1/2 (Ser217/221), phospho-p44/42 MAPK (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182; 1:1000), and corresponding HRP-conjugated secondary IgG antibodies (1:2000). All the antibodies were from Cell Signaling (Beverly, MA, USA). Proteins were visualized by chemiluminiscense (Super Signal West Pico chemiluminescent substrate, Pierce, Rockford, IL, USA) using a Kodak photo imager. β-Actin was detected as a loading control.

Quantitative PCR analysis of CCL6

RNA was purified from isolated eosinophils 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 μg/ml with diethylpyrocarbonate water. RNA was then reverse-transcribed to cDNA, and 1 μl of this cDNA was used in the *Taq*Man reaction mixture as described previously [15]. The specific primer/probe sets for real-time PCR were predeveloped by Applied Biosystems (Foster City, CA, USA).

Apoptosis/survival assays

Apoptotic cells were characterized by the inability to exclude trypan blue stain, loss of mitochondrial membrane potential, DNA degradation, and caspase-3 activation.

Viability assays

Eosinophils were cultured for 24 h with different treatments. Then, cells were resuspended carefully and incubated with trypan blue for 2 min at room temperature. Then, cell samples were counted using a hemocytometer. Dead cells were determined as trypan blue-positive.

Mitochondrial membrane potential

Following treatments, eosinophil cells were stained with JC-1 (10 μg/ml) at 37°C for 20 min. Following harvesting and washing with PBS, cells were resuspended in prewarmed (37°C) complete serum medium and analyzed. JC-1 aggregates were detectable in the fluorescence 2 (FL2) channel (red fluorescence, emission at 590 nm), and JC-1 monomers were detectable in the FL1 channel (green fluorescence, emission at 527 nm) [32]. The incorporation of JC-1 can be affected by factors other than mitochondria (e.g., cell size, plasma membrane permeability) [33]. To ensure that staining patterns were similar, cells (untreated and treated) were exposed to the K$^+$ ionophore valinomycin (100 nM) [34] for 15 min at room temperature and then analyzed again.

DNA degradation

The percentage of apoptotic cells was determined by analysis of subdiploid DNA (<2 N) analyzed by flow cytometry. The eosinophils were treated with 70% ethanol (100 μl/30,000 cells) for 10 min at 4°C. The cells were then washed twice in PBS and stained with propidium iodide (5 ng/ml, Sigma Chemical Co., St. Louis, MO, USA) at room temperature for 15 min. After incubation, the cells were diluted in flow buffer to give a final volume of 1 ml prior to analysis [35, 36].

Asp-Glu-Val-Asp (DEVD)ase activity

EnzChek caspase-3 assay kit #2 (Molecular Probes, Roche Applied Science, Indianapolis, IN, USA) was used to detect caspase-3 activity. Approximately 2 × 10^6 cells were collected by centrifugation and processed as we described before [31]. In brief, cell pellets were lysed in 50 μl lysis buffer using three freeze/thawing cycles and centrifuged at 5000 g for 5 min to pellet cellular debris. The resultant supernatant (50 μl) was transferred into a black polystyrene 96-well assay plate. To determine protein concentration by a modified Bradford assay, 1 μl resultant supernatant was used. After 30 min of incubation with reaction buffer (Z-DEVD-R110 substrate) at room temperature, the released fluorescence of the cleaved DEVD substrate was measured in a fluorescence plate reader (496 nm excitation and 520 nm emission). R110 was used as a standard curve. Data were expressed as μM cleaved caspase-3 normalized to mg protein.

ELISAs

Cytokines were quantified from supernatants of overnight-cultured cells using a standard sandwich antibody ELISA system. The murine ELISAs were set up using standardized antibodies purchased from R&D Systems (Minneapolis,

MN, USA), which detect protein at concentrations above 10 pg/ml, are specific, and do not cross-react with any other cytokines.

mRNA analysis of apoptosis-related proteins

Isolated eosinophils were cultured overnight with IL-5 (5 ng/ml), washed, and incubated with media with no IL-5 for 30 min. Eosinophils were then stimulated for 1 h with SCF (10 ng/ml), and the expression of genes related to apoptosis was analyzed. Briefly, immunoarrays containing the cDNA for the specific gene products were prehybridized in 4 ml Microhyb hybridization buffer (Research Genetics, Huntsville, AL, USA), containing 10 ml 10 mg/ml human Cot I DNA (denatured at 95°C for 5 min prior to use; Life Technologies, Gaithersburg, MD, USA) and 10 ml 8 mg/ml poly(dA) (denatured at 95°C for 5 min prior to use; Research Genetics). Total RNA (1–5 mg) was labeled with [33P]deoxy-cytidine 5′-triphosphate by RT for each time-point or treatment. After 4 h of prehybridization at 42°C, ~10^7 cpm/ml heat-denatured probe was added to each prehybridization mix, followed by 18 h of further incubation at 42°C in a roller oven (Hybaid, Waltham, MA, USA). Hybridized arrays were rinsed in 50 ml 2× SSC and 1% SDS twice at 55°C followed by one to two times of washing in 2× SSC and 0.1% SDS at 55°C for 10 min. The microarrays were exposed to phosphorimager screens for 1–3 days. The screens were then scanned in a PhosphorImager (Bio-Rad, Hercules, CA, USA) at 50 μm resolution. Gene expression was determined from experiments by capturing the pixel density (a.k.a., volume) of each spot in the Bio-Rad software.

Statistical analysis

Statistical significance was determined using ANOVA with P values less than 0.05 with a Student-Newman-Keuhl's post-test.

RESULTS

SCF-induced signal transduction pathways in eosinophils

Although some of the SCF-induced activation has been worked out in mast cells and other cell lines, none of the signal pathways have been established in eosinophils. Given the potential importance of SCF-induced eosinophil activation, it was imperative to first determine if the pathways are similar and second, describe whether the signaling pathways differentially activate the eosinophils. We initially began describing SCF signal pathways by demonstrating that we could detect tyrosine kinase activity after SCF stimulation of eosinophils. **Figure 1** illustrates that SCF induces tyrosine kinase activity beginning at 30 s and increased by 1 min and was further altered at 5 min poststimulation. In comparison with an eotaxin-induced signal, SCF appears to induce a similar signal with a number of proteins phosphorylated. Thus, these initial studies outline that SCF is an eosinophil-activating protein leading to significant intracellular signaling events.

In the next series of studies, we examined specific SCF-induced pathways. SCF stimulation of eosinophils induced activation of the PI-3K pathway assessed by examining AKT phosphorylation, which is activated directly by PI-3K (**Fig. 2A**). These studies further demonstrate that SCF induces a strong AKT signaling response comparable with eotaxin. SCF also induced prolonged activation of p38 MAPK kinase (Fig. 2B). Although the PI-3K and MAPK pathways have been shown to interact in several situations, it was important to also establish that they were both activated in response to SCF. Thus, numerous signaling pathways are activated by SCF in eosinophils.
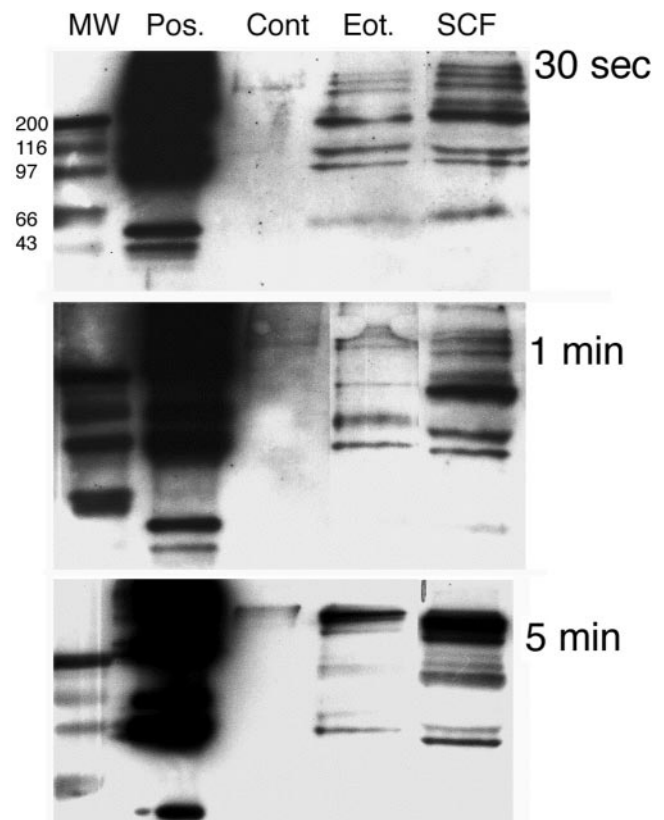

Fig. 1. Activation of phosphotyrosine in SCF-stimulated eosinophils. Freshly isolated murine eosinophils were synchronized overnight with IL-5, 5 ng/ml, washed, and then rested for 10 min and stimulated with eotaxin (10 ng/ml) or SCF (10 ng/ml) at 37°C. Harvested cells were lysed on ice. Tyrosine-phosphorylated proteins were detected by Western blotting in total cell lysate (2×10⁶ cells). Representative blots shown from three independent experiments. Pos., Positive control stimulated with PMA; Cont, untreated control; Eot., eotaxin.

MEK inhibitors have been used successfully to block ERK1 and ERK2 activation in fMLP-activated, IL-5 primed eosinophils [37, 38]. We have observed activation of MEK1/2 and downstream phosphylation of p44/42 MAPK by SCF stimulation within the first 10 min in isolated eosinophils (**Fig. 3A**). To investigate SCF-induced signaling further, we blocked Akt and MEK1/2 phosphorylation using specific inhibitors, Wortmannin and U0126, correspondingly (Fig. 3B). As depicted in Figure 3B, SCF-induced Akt activation was not altered by U0126. Thus, these pathways appeared to be activated independently by SCF.

Blocking of MEK1/2 significantly reduces SCF-induced CCL6 expression

Next, as our previous data had demonstrated that SCF activates a number of inflammatory mediators, we were interested in whether specific pathways were responsible for activation of certain inflammatory genes. In particular, chemokines can be shown to be up-regulated by SCF. CCL6 (C10) has been shown previously to be highly expressed in eosinophils [39] and can be up-regulated significantly by SCF, assessed by quantitative PCR after 1 h of SCF stimulation (**Fig. 4A**). It is also important to note that our studies with macrophage and polymorphonu-
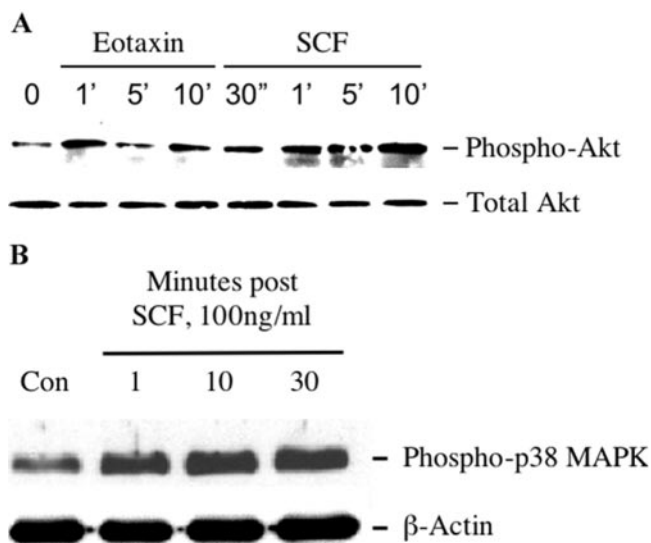
Fig. 2. SCF activation of eosinophils induces phosphorylation of Akt (A) and p38 MAPK (B). Freshly isolated murine eosinophils were synchronized overnight with IL-5, 5 ng/ml, washed, and resuspended in serum-free media and stimulated with eotaxin (10 ng/ml) or SCF (10 ng/ml, A). Harvested cells were lysed on ice, and detergent-soluble fractions were separated by 12% SDS-PAGE and then analyzed by Western blotting with phospho-Akt or phospho-p38 MAPK. Total Akt or β-actin was detected as a loading control. Representative blots shown from three to five independent experiments. Con, Untreated control.

clear neutrophil populations, which were the primary contaminant (1–2%) in our preparations, have demonstrated no expression of c-kit and no activation with SCF (data not shown). Next, we used the inhibitors, described previously, of the signaling pathways to assess the influence of the different pathways on gene activation in the isolated eosinophils. SCF-induced CCL6 mRNA expression was reduced significantly (2.52-fold) by MEK1/2 inhibition with U0126. The same trend of data was observed with CCL6 chemokine production after 24 h incubation, as assessed in the supernatants by specific ELISA (Fig. 4B). However, when we inhibited PI-3K signaling using Wortmannin, no such inhibition was observed, suggesting that CCL6 activation was not mediated via the PI-3K arm of the signaling cascade (Fig. 4). Changes in CCL6 mRNA expression level and chemokine production after eosinophil treatment with U0126 and SCF (Fig. 4) correlated with the MEK1/2 activation levels observed by Western blot (Fig. 3B). Thus, it appears that the CCL6 gene activation was dependent on the MAPK pathway in SCF-stimulated eosinophils.

SCF-induced eosinophil survival was abrogated by blocking MEK with U0126

One of the primary roles of SCF in mast cell biology is its ability to promote survival. Therefore, the role of SCF in preventing eosinophil apoptosis was investigated. We have chosen multiple methods to detect progression to cell death: count of viable cells by trypan blue exclusion, propdium iodide staining, membrane potential changes, and caspase release assays. The enumeration of viable cells by trypan blue exclusion showed that SCF treatment increased eosinophil survival

over untreated control, even at the lowest dose examined (**Fig. 5A**). Freshly isolated eosinophils were cultured and synchronized overnight with IL-5 (5 ng/ml). Cells were subsequently cultured in the fresh media without IL-5 and treated with SCF (1, 10, or 100 ng/ml) for 24 h. SCF activation demonstrated that 1 ng/ml SCF was sufficient to increase eosinophil survival significantly compared with cells cultured with no SCF, and 10 ng/ml increased survival further, and higher levels had no additional benefit, as assessed by trypan blue exclusion analysis (Fig. 5A). Next, we inhibited the two distinct pathways, MEK and PI-3K, using U0126 or Wortmannin, respectively (Fig. 5B). The assessment of eosinophil survival by trypan blue demonstrated that inhibition of MEK in the presence of SCF blocked increased survival, whereas Wortmannin had no effect. In addition, in comparison with IL-5 alone (5 ng/ml), an established eosinophil survival factor, SCF appeared to have similar efficacy for increasing survival. It is important that the inhibitors incubated with eosinophils on their own demonstrated no reduction in eosinophil survival compared with control cultures. Thus, similar to the role of SCF in other cell populations, SCF appears to promote eosinophil survival.
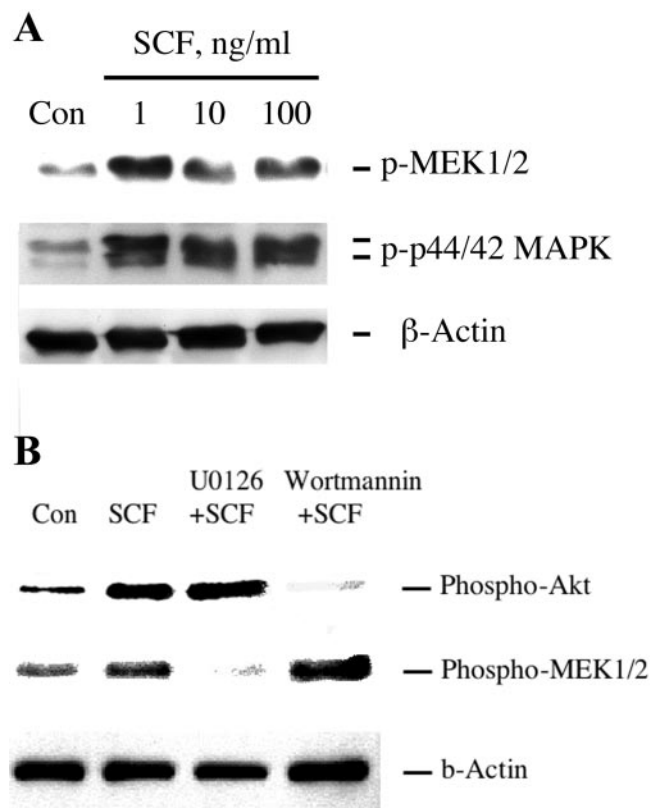

Fig. 3. SCF activation of eosinophils induces phosphorylation of MEK1/2 and p44/42 MAPK (p-MEK1/2 and p-p44/42 MAPK, respectively; A), and Wortmannin and U0126 specifically block phosphorylation of Akt and MEK1/2, respectively (B). Freshly isolated murine eosinophils were synchronized overnight with IL-5, 5 ng/ml, and then resuspended in serum-free media and stimulated with SCF for 10 min or pretreated for 10 min with Wortmannin (1 μM) and U0126 (10 μM) at 37°C. Harvested cells were lysed on ice, and detergent-soluble fractions were separated by 12% SDS-PAGE and analyzed by Western blotting with phospho-MEK1/2-, phospho-p44/42 MAPK-, or phospho-Akt-specific antibodies. β-Actin was detected as a loading control. Representative blots shown from three independent experiments.
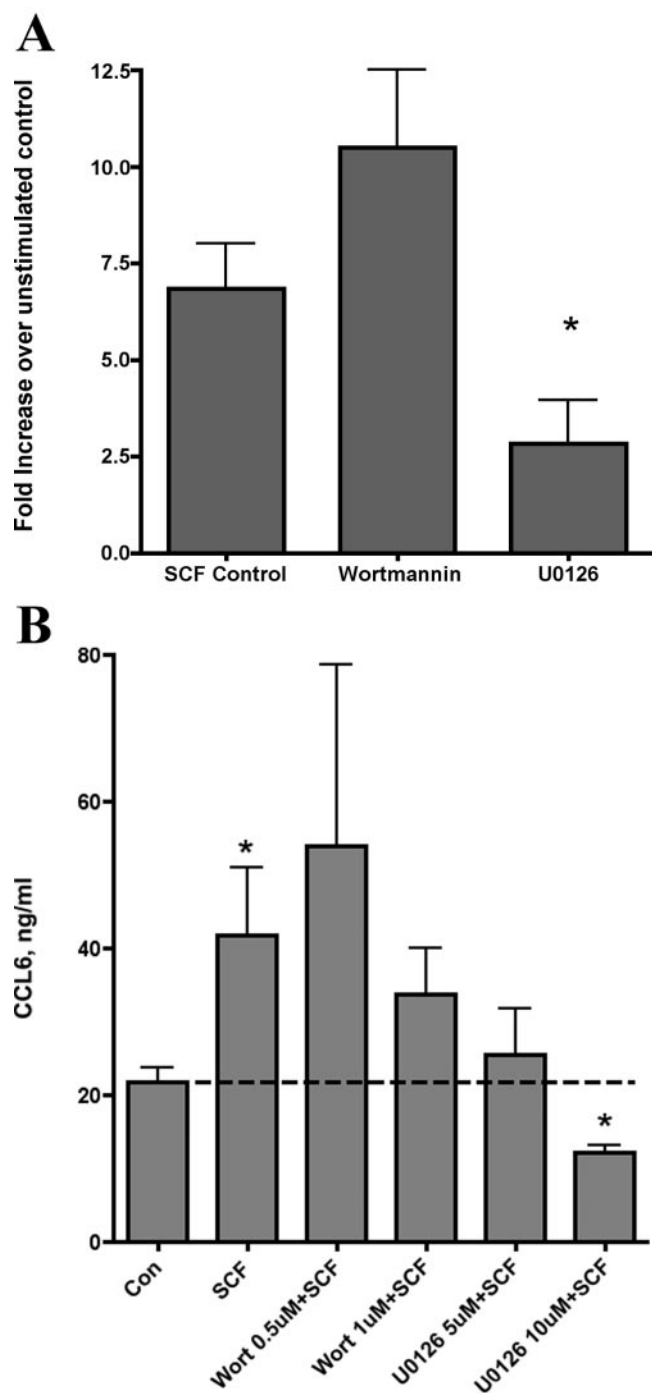
Fig. 4. U0126 (MEK1/2) inhibitor but not Wortmannin (PI-3K) suppressed CCL6 mRNA expression and chemokine production after SCF activation. Eosinophils were pretreated with U0126 (10 μM) or Wortmannin (1 μM) for 10 min and then stimulated with SCF, 100 ng/ml, for 1 h at 37°C and subjected to real-time PCR analysis for the expression of CCL6 (A). Eosinophils were pretreated with U0126 (10 μM) or Wortmannin (1 μM) for 10 min and then stimulated with SCF, 100 ng/ml, for 24 h at 37°C. Supernatants were collected and assayed for a CCL6 level using a specific ELISA (B). Data for each group are the mean ± SE from three different experiments. *, Significance at $P < 0.05$.

Analysis of mitochondria membrane potential by flow cytometry for assessment of cell survival, as indicated by high mitochondrial membrane potential, confirmed the previous set of experiments. As shown on Figure 5C, eosinophils incubated without any SCF had a high level of spontaneous apoptosis, as indicated by a significantly lower level of mitochondrial membrane potential compared with control eosinophils at time 0 (Con "0"). Administration of SCF (10 ng/ml) increased eosinophil survival significantly, from 43.7% to 71.7% (Fig. 5C). Pretreatment with U0126 (10 μM) eliminated SCF-dependent inhibition, leading to only 25% of cells surviving, as indicated by low membrane potential (Fig. 5C). It is important that mitochondrial membrane potential was not reduced further with U0126 on its own in control, unstimulated cultures. The use of the PI-3K inhibitor Wortmannin only slightly reduced the survival promoted by SCF, which was not significant.

Analysis of DNA content by flow cytometry confirmed the previous sets of experiments. All cells with DNA content less then 2 N were counted as apoptotic or necrotic. As shown on Figure 5D, eosinophils have a high level of spontaneous apoptosis. Administration of SCF (100 ng/ml) reduced eosinophil apoptosis significantly from 37.2% to 12.6%, maintaining eosinophil survival at a similar level, as observed with viable eosinophils prior to the incubation at Time 0, i.e., control, fresh cells (Fig. 5D). Pretreatment with U0126 (10 μM) eliminated the SCF-dependent survival, leading to 56.3% of cells with degraded DNA (Fig. 5D). Wortmannin-treated cells (1 μM), prior to SCF, did not abrogate SCF-mediated eosinophil survival (Fig. 5D).

Qualitative data observed by flow cytometry techniques were confirmed by quantitative caspase-3 activity assays (Fig. 5E). It is known that activation of caspase-3 in eosinophils is a terminal step, which leads to apoptosis [40]. SCF treatment (10 ng/ml) of cells resulted in 42.4% inhibition of caspase-3 activity. Combined treatment with Wortmannin (0.5 or 1 μM) plus SCF did not reduce the SCF-mediated effect on caspase-3 activity significantly. In contrast, pretreatment of cells with U0126 (1 or 10 μM) resulted in an increase in caspase-3 activity, reaching a 2.18-fold increase over untreated control at a 10-μM dose of U0126 (Fig. 5E). As a control, staurosporin induced extremely high levels of caspase-3, and all of the eosinophils became apoptotic. Altogether, it appears that although the PI-3K-mediated pathway was activated, the MEK pathway appeared to be the primary pathway involved in SCF-mediated eosinophil survival.

SCF induces expression of antiapoptotic genes

An important area that has been established during the development of apoptosis is the differential expression of a group of genes that are members of the BCL family. The Bcl-2 family is now recognized as central in the control of apoptosis in many cell types, and this family can be divided into two groups: antiapoptotic proteins and proapoptotic proteins. The relative abundance of these proteins is thought to control the commitment of a cell into apoptosis or survival [41]. We have analyzed the expression of a number of these Bcl family members and found that although SCF up-regulates Bcl-2 and Bcl-3 significantly (**Fig. 6**), it is known that Bcl-3, in complex with p52, can promote transcription of the genes encoding the cell cycle regulator cyclin D1 and the antiapoptotic Bcl-2 protein [42]. Some of these genes have been shown to promote apoptosis, and other family members have clearly been identified as antiapoptotic. We have analyzed the expression of a number of these BCL family members and found
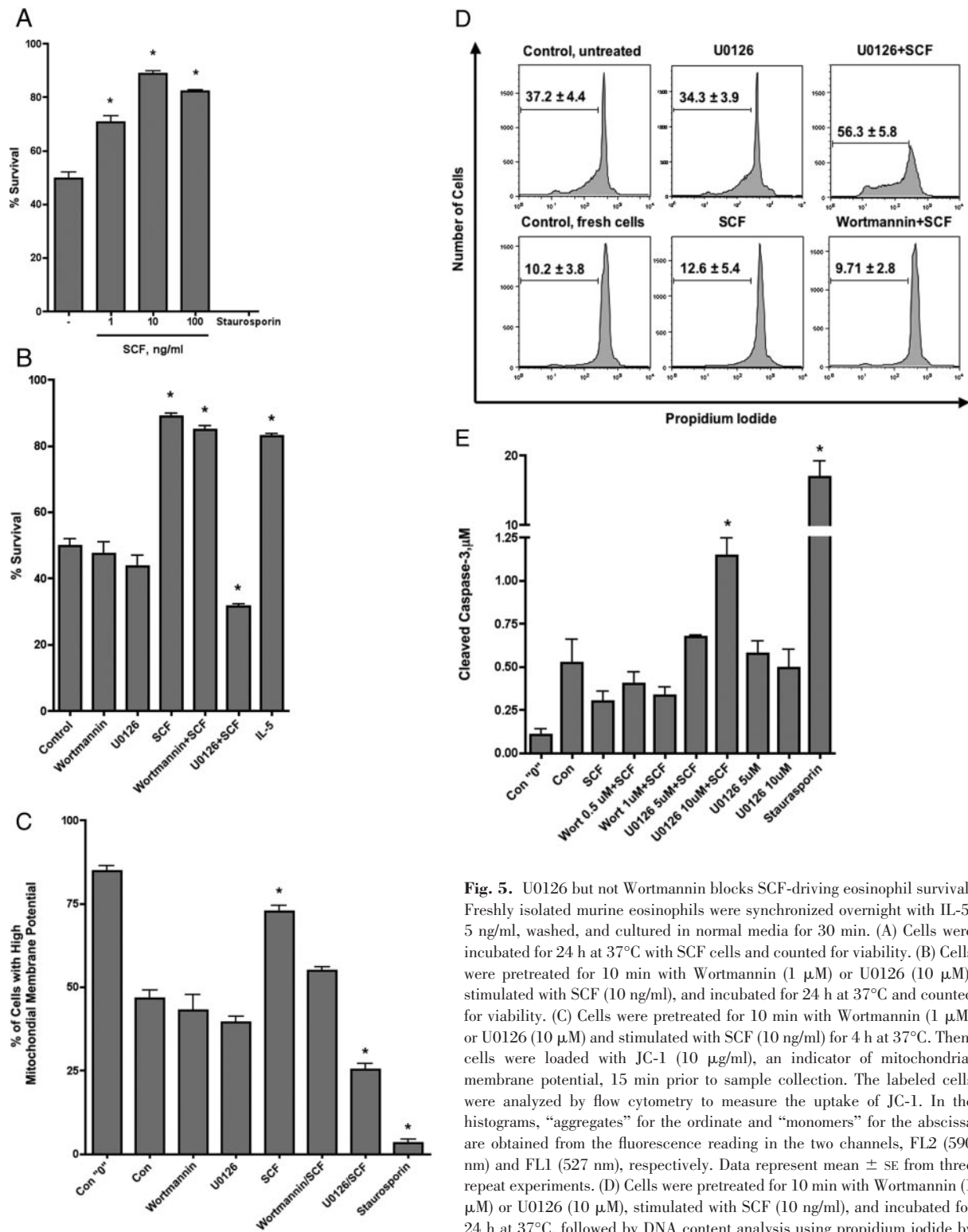
Fig. 5. U0126 but not Wortmannin blocks SCF-driving eosinophil survival. Freshly isolated murine eosinophils were synchronized overnight with IL-5, 5 ng/ml, washed, and cultured in normal media for 30 min. (A) Cells were incubated for 24 h at 37°C with SCF cells and counted for viability. (B) Cells were pretreated for 10 min with Wortmannin (1 μM) or U0126 (10 μM), stimulated with SCF (10 ng/ml), and incubated for 24 h at 37°C and counted for viability. (C) Cells were pretreated for 10 min with Wortmannin (1 μM) or U0126 (10 μM) and stimulated with SCF (10 ng/ml) for 4 h at 37°C. Then, cells were loaded with JC-1 (10 μg/ml), an indicator of mitochondrial membrane potential, 15 min prior to sample collection. The labeled cells were analyzed by flow cytometry to measure the uptake of JC-1. In the histograms, "aggregates" for the ordinate and "monomers" for the abscissa are obtained from the fluorescence reading in the two channels, FL2 (590 nm) and FL1 (527 nm), respectively. Data represent mean ± SE from three repeat experiments. (D) Cells were pretreated for 10 min with Wortmannin (1 μM) or U0126 (10 μM), stimulated with SCF (10 ng/ml), and incubated for 24 h at 37°C, followed by DNA content analysis using propidium iodide by flow cytometry. (E) Cells were pretreated for 10 min with Wortmannin (0.5 and 1 μM) and U0126 (5 and 10 μM) and/or stimulated with SCF, 10 ng/ml, for 16 h at 37°C. Caspase-3-like activity was assessed according to the manufacturer's instructions. Data for each group are the mean ± SE from two different experiments. *, Significance at $P < 0.05$, compared with untreated control; **, significance at $P < 0.05$, compared with SCF-treated, positive control.

that although SCF up-regulates BCL2, MCL-1, and BCL3 significantly, other proapoptotic genes, including BAD and BAK1, were not up-regulated compared with control eosinophils (Fig. 6). Thus, the expression of this gene profile after SCF is consistent with the findings above, demonstrating that SCF promotes survival for eosinophils.

DISCUSSION

The activation and degranulation of eosinophils may be the causative events related to the exacerbation of airway disease [43–45]. In particular, eosinophils have been reported to be the primary cells associated with induction of bronchial mu-
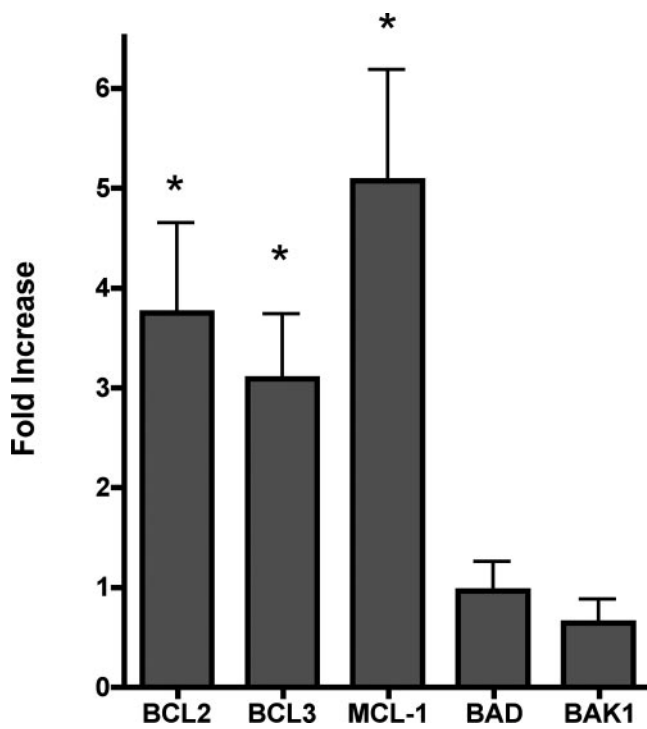
Fig. 6. SCF activates expression of prosurvival genes and blocks apoptotic genes. Freshly isolated murine eosinophils were synchronized overnight with IL-5, 5 ng/ml, and then cultured in media without IL-5 for 30 min and treated with SCF, 10 ng/ml, for 1 h. Isolated mRNA was then subjected to mRNA gel blot analysis using P33 labeling and subjected to densitometry analysis using a Bio-Rad phosphoimager. The fold increase over control was calculated from three repeat experiments comparing SCF-stimulated eosinophils with control, unstimulated cultures. BCL2, MCL-1, and BCL3 expression was increased significantly: *, $P < 0.05$.

cosal injury and are thought to participate in bronchial obstruction and airway hyper-reactivity [5, 46]. In fact, recent studies using IL-5-deficient mice or mice treated with anti-CCR3 demonstrated that there was an abrogation of airway hyper-responsiveness and mucus production after an allergen challenge related directly to the decrease in eosinophil accumulation [47, 48]. In the present studies, we have shown that SCF activates eosinophils for CCL6 production and cell survival via the MAPK but not PI-3K pathway. Although it is possible that the effects that we observed with decreased CCL6 production with the MEK inhibitor may be attributed to decreased survival, the expression of CCL6 mRNA at 1 h suggested that it has an immediate effect on SCF-induced gene transcription. Numerous SCF-mediated genes have been explored in progenitor cell populations and relate to the maturation of various cell populations in combination with other hematopoietic factors. In addition, other investigations have identified the importance of the SCF-mediated MAPK pathways in mast cell proliferation [24, 49]. SCF has also been implicated in the migration of mast cells and most recently, identified that it is the PI-3K-mediated pathways and not the MAPK pathways that are necessary during VLA-4 cooperative movement [50–52]. We did not investigate the differential role of these two signaling pathways in eosinophil migration, but it would be interesting in future studies to address this issue. It

may be especially intriguing to examine the role of SCF for enhancing chemokine-mediated eosinophil migration.

The assessment of apoptotic pathways was accomplished using multiple methods of detection. It is interesting that several parameters of survival and apoptosis appear to indicate that SCF-induced eosinophil survival is a function of MEK activation. The eosinophil apoptosis induced by dexamethasone has been associated with caspase-3 activity [53]. In contrast, it was reported at the same time that inhibition of caspase-3 activity failed to block glucocorticoid or Fas ligand-induced eosinophil apoptosis [40, 53]. It was suggested that proteolytic caspase-3 activity is diminished in eosinophils, perhaps as a result of inadequate processing of the procaspase [40]. Conversely, it has been shown that spontaneous human eosinophil apoptosis involves caspase-3 activity [54]. Here, we have shown that SCF was able to reduce caspase-3 activity related to spontaneous mouse eosinophil apoptosis. Pretreatment with U0126 but not with Wortmannin resulted in a significant increase in caspase-3 activity. The increase in eosinophil survival also correlated with increases in BCL2, BCL2-related MCL-1, and BCL3, which have been identified as antiapoptotic proteins. BCL2 and another BCL2 family member MCL-1 have been shown to inhibit eosinophil apoptosis and prolong eosinophil survival [55–58]. Although this is the first evidence for the expression of increased BCL3 related to survival in eosinophils, other data have suggested that BCL3 has an important role in regulation of Bim in activated T cells directly related to survival and could play similar roles in eosinophils [59]. Although eosinophil apoptosis in many allergic disorders is attenuated by IL-5 [40, 54, 56, 60–62], the novel data in this study demonstrate that SCF was also effective for eosinophil survival. In addition to the activation parameter induced by SCF, its ability to provide a survival signal may be important for establishing chronic disease. Although SCF is an important mast cell survival factor [63], the antiapoptotic function of SCF may help explain previous observations that demonstrate that blockade of pulmonary SCF reduced the eosinophil presence in the lung upon allergen challenge [14–16, 64]. It appears that the antiapoptotic function of SCF depends predominantly on MEK1/2 activation. This may be similar to the IL-5 pathway, which also depends on a MAPK pathway for eosinophil survival [65, 66]. Furthermore, IL-5 and SCF activation pathways have been cooperatively linked via MAPK and PI-3K pathways, which promoted cell survival in TF-1 and JYTF-1 cell lines associated with Mcl-1 expression [67].

There are a number of allergic diseases associated with tissue eosinophil accumulation and activation, leading to tissue damage [68–70]. The current antieosinophil therapies are designed to inhibit eosinophil maturation and/or release of eosinophil-derived products. The therapeutic agents include glucocorticoids, myelosuppressive drugs, tyrosine kinase inhibitors, IFN-α, and humanized anti-IL-5 antibodies [6]. Some of these inhibitors have significant drawbacks. Treatment with glucocorticoids may limit useful T cell responses including respiratory tolerance and T-regulatory cell development [71]. Anti-IL-5 therapy for asthma may be limited because of the redundant pathways [6]. Together, the present studies offer additional understanding of the importance of the SCF-mediated pathway for the activation and survival of eosinophils.

The cellular source of SCF within the allergic airway may designate those cells susceptible to eosinophil activation products. In particular, airway epithelial cells and smooth muscle cells as well as pulmonary fibroblasts have been reported to express SCF [72–78], which subsequently can be cleaved from these cells during the inflammatory responses. However, as SCF is maintained initially as a membrane-bound protein, it may provide a solid-phase activation system to maintain the eosinophils that accumulate in and around the airways, long-term, without other survival factors such as IL-5. In support of SCF having a pathogenic role in chronic airway responses are studies demonstrating that the neutralization of SCF within the airway alleviates disease pathogenesis. Pharmacological inhibition of c-kit receptor tyrosine kinase with imatinib or intratracheal administration of specific anti-SCF in our chronic asthma murine model attenuated airway hyper-reactivity, peribronchial eosinophils, mucus overexpression, and remodeling [15, 16, 64, 79, 80]. These latter studies indicate that SCF would impact on the progression of the allergic responses directly. These present studies begin to outline the signaling mechanisms that are involved in SCF-mediated eosinophil activation as well as demonstrate, for the first time, that SCF/c-kit activation can promote eosinophil survival.

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