Stem cell factor and interleukin-2/15 combine to enhance MAPK-mediated proliferation of human natural killer cells

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Introduction

Stem cell factor (SCF; also known as c-kit ligand, steel factor, and mast cell growth factor) synergizes with hematopoietic growth factors such as erythropoietin,1 GM-CSF,2 and G-CSF,3 to promote lineage-specific, hematopoietic precursor cell growth. Interleukin-15 (IL-15) mediates differentiation of CD34dimCD45RA– hematopoietic precursor cells (HPCs) into human natural killer (NK) cells. This can be mimicked by IL-2 signaling through the same βγ receptor complex.5,6 The high-affinity IL-2 receptor and c-kit are both expressed from the earliest IL-15-responsive NK-cell precursor in humans through the fourth stage of NK-cell differentiation.7 In combination with IL-2/15, SCF enhances growth of CD34dimCD45RA+ integrin β7+ high hematopoietic precursor cells (HPCs) into human natural killer (NK) cells. This can be mimicked by IL-2 signaling through the same βγ receptor complex.5,6 The relevance of c-kit whose kinase activity is required for NK-cell proliferation and NK-cell development,9 MAPK-ERK signaling is stimulated through the binding of extracellular growth factors such as SCF and IL-2/15 to their cognate transmembrane receptors.10 The phosphorylated intracytoplasmic tail of the receptor in turn recruits Grb2 that binds guanine exchange factor son of sevenless (SOS) at the membrane. SOS then interacts with H-ras that in turn leads to its activation via guanosine triphosphate (GTP) loading followed by the sequential recruitment and activation of Raf, MAP/ERK kinase (MEK), and ERK. MEK binds and restricts inactive ERK to the cytosol. MEK and ERK complex in the cytosol and then dissociate when MEK is activated and phosphorylates ERK.11 Activated ERK then dimerizes and translocates to the nucleus and induces gene expression required for cell proliferation, development, and survival.10

In this report, we uncover the molecular mechanism explaining, at least in part, the observed enhancement in proliferation for the NK lineage in the presence of SCF and IL-2/15 compared with either cytokine alone.

Methods

Antibodies and inhibitors

Monoclonal antibodies (mAbs) specific to phospho-MEK (no. 9121), phospho-Erk1/2 (no. 9101), p27 (no. 2552), and the MEK1/2-specific kinase inhibitor U0126 (no. 9903) were purchased from Cell Signaling Technology (Beverly, MA). The c-kit tyrosine kinase inhibitor AG1296 (no. 658551) was obtained from Calbiochem (San Diego, CA). The CDK4-specific inhibitor, PD032991, was obtained from Pfizer Pharmaceuticals


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proliferation of primary, human CD56bright NK cells is seen in similar findings as seen in panel A. (C) Modest synergistic
mL), IL-2 (150 IU/mL), or SCF (100 ng/mL), IL-2 (150 IU/mL), or SCF + IL-2 for 48 hours. (B) IL-15 (20 ng/mL) is substituted for IL-2 and leads to similar findings as seen in panel A. (C) Modest synergistic proliferation of primary, human CD56bright NK cells is seen in response to SCF + IL-2 compared with SCF (250 ng/mL) or IL-2 (150 IU/mL) alone as originally described. All pairwise comparisons are statistically significant between treatment groups (P < .05) in panels A-C, and each figure shows combined data from at least 3 independent experiments.

Cell line and primary NK-cell preparation

The IL-2–dependent CD3−CD56+ NK-cell line DERL-712 (a generous gift from R. Di Noto and CEINGE Biotechnologie, Naples, Italy) was maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 20% FCS (Invitrogen), antibiotics (Invitrogen), and 150 IU/mL IL-2 (Hoffman-LaRoche, Nutley, NJ). The NK-cell line DERL-7 has been shown to be phenotypically and functionally similar to the human CD56bright subset.12 The major functional difference between the cell line and primary human NK cells is that the DERL-7 line exhibits a modest proliferative response to SCF alone, whereas this effect is not seen in primary human NK cells.

All work associated with human cells was approved by The Ohio State University Institutional Review Board. Human NK cells were isolated from peripheral blood leukopacks of healthy subjects (American Red Cross, Columbus, OH) and maintained as previously described.13 Briefly, NK cells were prepared and cultured as described in the presence of appropriate cytokines and growth factors.

Flow cytometric–based cell-cycle analysis

Cells were prepared and cultured as described in the presence of appropriate cytokines. Cells were harvested and prepared in a single-cell suspension buffer. After 2 washes, cells were resuspended in ice-cold ethanol and kept at −20°C until analysis. Propidium iodide staining solution (50 µg/mL) was added with RNase A stock solution (20 µg/mL). Data were collected using DNA QC software (BD Biosciences) according to the manufacturer’s protocol, running on a FACSCalibur Flow Cytometer (BD Biosciences), and were analyzed using CellQuest software (BD Biosciences).

Retroviral infection of the DERL-7 NK cell line

Retroviral infections of the NK-cell line DERL-7 were performed using a MIGR1 retroviral vector following previously published protocols.14,15 Briefly, infectious supernatants from MIGR1, MIGR1-ERK1, and MIGR1-ERK2 (provided by D.P.) transiently transfected Phoenix cells were collected 48 hours after transfection and used for 3 cycles of infections.
Upon infection, DERL-7 cells were sorted for green fluorescent protein (GFP) expression. Overexpression of ERK1 or ERK2 was confirmed in the DERL-7 CD56\(^{+}\)/H11001/GFP\(^{+}\)/H11001 cells by Western blotting.

**Statistical considerations**

For intergroup comparisons between cytokine-stimulated conditions in proliferation assays, one-way analysis of variance (ANOVA) was used to assess group effects with subsequent multiple comparisons by Tukey method. In some circumstances, a linear mixed effects model was applied to fit data such that repeated measures could be accounted for properly across multiple experiments. In experiments where an a priori specified comparison was examined, a Bonferroni correction (alpha for each test was set to .016) was used. Fold change in mean fluorescent intensity (MFI) of CD117 surface expression in response to cytokine stimulation was assessed by one-sample \( t \) test from results of 5 independent experiments.

**Results**

**Costimulation with SCF and IL-2/15 leads to synergistic proliferation of NK cells**

We first confirmed that the CD56\(^{bright}\) NK-cell line DERL-7 displays a modest amount of proliferative synergy in response to culture with SCF and IL-2 (Figure 1A) or with SCF and IL-15 (Figure 1B). In all circumstances, proliferation is measured percentage above that of untreated controls. Cells cultured in serum-free medium (SFM) with SCF (100 ng/mL) alone for 48 hours induced 96% plus or minus 2.2% (SEM) growth, whereas IL-2 (150 IU/mL) induced 206% plus or minus 5.2% growth, and the combination resulted in 360% plus or minus 8.6% growth. As shown in Figure 1B, as the IL-2/15R complex is activated in response to either cytokine, substitution of IL-15 for IL-2 yields a similar phenomenon. After 48 hours, SCF (100 ng/mL) yielded 29% plus or minus 4.5% growth, IL-15 (20 ng/mL) induced 206% plus or minus 5.2% growth, and the combination resulted in 360% plus or minus 8.6% growth. Data shown are combined from 3 independent experiments, demonstrating mean percentage proliferation (± SEM); AG1296 virtually abolished any SCF-dependent proliferation (left bars, \( P < .01 \)) and a modest impact was seen on IL-2–mediated proliferation (center bars, \( P < .01 \)). The main finding (comparison indicated by * in panel B) is that the sum total contribution of SCF to synergistic proliferation was abrogated in the presence of AG1296 (\( P < .01 \)), as proliferation in response to IL-2 was identical to that of SCF + IL-2 (\( P = n/s \)).
IL-2 stimulation up-regulates surface density expression of c-kit (CD117)

Based on data from other models of growth factor synergy involving SCF, we hypothesized that proliferative synergy between SCF and IL-2 may be related to cell surface receptor expression, per se.1,2 DERL-7 cells starved for 48 hours in SFM were then stimulated with SCF, IL-2, or SCF plus IL-2 and surface density expression of c-kit as well as the IL-2/15R β and γ subunits was assessed by flow cytometry. IL-2 stimulation led to a statistically significant up-regulation of CD117 (c-kit) expression (Figure 2A). In 5 independent experiments, the mean fluorescent intensity (MFI) of CD117 was 73 on resting cells and 160 on cells stimulated with IL-2 (mean fold change = 2.1, \( P < .001 \)). Interestingly, the reciprocal induction of IL-2/15Rβ and γ gene expression following culture in SCF (or SCF + IL-2) was not observed in the primary human NK cells or the NK-cell line DERL-7 (data not shown).

The tyrosine kinase activity of c-kit is required for SCF-induced proliferation as well as proliferative synergy

We hypothesized that up-regulation of CD117 surface expression through activation of the IL-2/15R serves to facilitate the proliferative synergy between SCF and IL-2/15. We therefore investigated whether a functional consequence of up-regulated CD117 expression might occur. The DERL-7 NK cell line was starved for 48 hours in SFM and then incubated for 2 hours in DMSO (vehicle) or AG1296 (5 μM), a tyrosine kinase inhibitor specific for c-kit.2 By Western blotting, DERL-7 cells incubated in SCF have increased levels of phospho-ERK compared with unstimulated Derl-7 cells, and this is abolished by the c-kit inhibitor, AG1296. DERL-7 cells incubated in IL-2 have increased phospho-ERK compared with unstimulated Derl-7 cells, and this is not affected by

### Figure 3. Phosphorylated ERK is uniquely increased in response to SCF + IL-2 as opposed to SCF or IL-2 alone.

(A) The top panel shows Western blot results from starved DERL-7 cells stimulated with SCF, IL-2, or SCF + IL-2, suggesting an increase in phosphorylated ERK in response to the combination of cytokines compared with either alone. Actin serves as a loading control. On the bottom, band densitometry confirms that composite mean (± SEM) phospho-ERK levels (relevant pairwise comparisons indicated by \( * \) in panel A, \( P < .05 \)) from 3 independent experiments. (B) Similar findings are observed in primary human NK cells. Once again, the top panel shows Western blot results for phosphorylated ERK in response to SCF, IL-2, or the combination. On the bottom, band densitometry confirms that composite mean (± SEM) differences from 4 independent experiments shown are also statistically significant (relevant pairwise comparisons indicated by \( * \) in panel B, \( P < .05 \)).

### Figure 4. IL-2-mediated NK-cell proliferation as well as SCF + IL-2 proliferative synergy are MAPK dependent.

For panels A and B, the y-axis represents the mean percentage proliferation (± SEM) above that measured for untreated controls as measured by the MTS assay. (A) Primary, human NK cells pretreated with either UO126 (10 μM) or DMSO (vehicle) were cultured in SCF, IL-2, or SCF + IL-2. Combined data from 3 independent experiments show mean proliferation (± SEM) in response to IL-2 and in response to SCF + IL-2 was decreased in the presence of the MAPK inhibitor compared with vehicle control (indicated by \( * \) in panel A, \( P < .05 \) for each comparison shown). (B) Overexpression of ERK1 and ERK2 in the NK-cell line DERL-7 yields complementary results. Overexpression of ERK1 (\( * \)) and ERK2 (\( ** \)) led to statistically significant increases in proliferation (± SEM) in response to IL-2 and SCF + IL-2 (comparisons indicated by \( * \) respectively in panel B, \( P < .05 \) for all indicated comparisons) compared with cells treated with vector alone (\( \circ \)).
Figure 5. MAPK-mediated synergistic proliferation is mediated in part by unique modulation of the G1/S cell-cycle checkpoint. (A) The NK-cell line DERL-7 is more than 95% viable by trypan blue staining and is entirely in G0 after 48 hours in SFM (left panel). After 24 hours in SCF, IL-2, or SCF + IL-2, increasing proportions of cells transit beyond G1/S. SCF + IL-2–stimulated cells (right panel) have the highest proportion beyond the G1/S checkpoint ($P < .05$). Statistically significant results are representative of 3 independent experiments. (B) Starved DERL-7 cells were stimulated with SCF, IL-2, or SCF + IL-2 for 18 hours in the presence of DMSO (carrier) or an inhibitor of MAPK. Top: Immunoblotting for p27 in the top left panel reveals that, in the presence of DMSO, loss of p27 is greater in response to SCF + IL-2 than in response to IL-2 or SCF alone. The bottom panel shows actin as a loading control. Immunoblotting for p27 in the top right panel reveals that, in the presence of the MAPK inhibitor U0126, there is no change in p27 expression. Bottom left: The bar graph summarizes band densitometry data shown as the composite mean (± SEM) from 3 independent immunoblotting experiments such as that shown on the top left in the presence of DMSO. It confirms that these differences in p27 expression are statistically significant (comparisons indicated by * in panel B, $P < .05$ for all pairwise comparisons). Bottom right: The bar graph summarizes band densitometry data shown as the composite mean (± SEM) from 3 independent immunoblotting experiments such as that shown on the top right in the presence of the MAPK inhibitor U0126. It confirms that these differences in p27 expression are not...
the presence of the c-kit inhibitor, AG1296. DERL-7 cells incubated in SCF plus IL-2 have increased phospho-ERK levels that are greater than the phospho-ERK levels seen in the presence of IL-2 or SCF alone. DERL-7 cells incubated in IL-2 plus SCF in the presence of AG1296 have phospho-ERK levels that are similar to cells treated with IL-2 alone (data not shown). Cells were then cultured in SFM, SCF, IL-2, or SCF plus IL-2 for 48 hours, followed by a 2-hour measurement of proliferation. Combined data from 3 independent experiments in the NK-cell line DERL-7 are shown in Figure 2B, demonstrating that inhibition of c-kit tyrosine kinase activity abolishes the sum total contribution to the modest proliferative synergy in the SCF plus IL-2 condition. Culture in SCF alone led to 93% plus or minus 5.2% growth in vehicle (dimethyl sulfoxide [DMSO]) versus unstimulated cells in SFM. However, in the presence of AG1296, the proliferative response was virtually abolished, measuring only 0.5% plus or minus 0.7% of untreated controls (P < .001). Culture in IL-2 alone led to 234% plus or minus 6.2% growth that was partially suppressed to 163% plus or minus 3.5% in the presence of AG1296 (P < .05).

The mechanism of this latter observation is somewhat unclear. We verified that the SFM contained no SCF. We also verified that starved, resting cells had no evidence of phosphorylation of c-kit kinase. As IL-2 stimulation, per se, significantly up-regulates surface expression of available c-kit (as shown previously in Figure 2A), this receptor expression amplification could potentially lead to activation of c-kit in the absence of ligand. However, we did not observe direct phosphorylation of c-kit following stimulation with IL-2. Indeed, we did not observe direct transphosphorylation events between these cytokine receptors following stimulation with either SCF or IL-2 (data not shown). In other words, IL-2/IL-15 stimulation did not result in c-kit kinase phosphorylation nor did SCF stimulation result in phosphorylation of the IL-2/15R. Another explanation could be that AG1296 is less specific as a c-kit kinase inhibitor than previously described. Consistent with this idea is the prior observation that AG1296 impairs growth in response to lineage-specific cytokine stimulation in other models of SCF growth factor synergy.

We also examined the physical relationship of the IL-2/15R heterodimeric complex and c-kit. Previous groups have shown that elements of other lineage-specific cytokine receptors physically colocalize with c-kit to facilitate proliferation. In fact, using a coimmunoprecipitation/Western blot technique, we observed reciprocal colocalization between c-kit and the IL-2/15Rβ chain (data not shown), but we could not demonstrate such colocalization between IL-2/15Rγ and c-kit.

The key finding in this set of experiments was that c-kit kinase activity appeared necessary for SCF-enhanced IL-2/15 NK-cell proliferation to occur. Proliferation in response to SCF plus IL-2 in DMSO was 357% plus or minus 7% above untreated controls, whereas the addition of AG1296 allowed IL-2 plus SCF–mediated proliferation to increase by only 185% plus or minus 3.2% above untreated controls (P < .01). In other words, the entire functional contribution of SCF stimulation to proliferative synergy with IL-2 seems to be dependent upon intact c-kit tyrosine kinase activity.

**MAPK signal transduction is uniquely modulated by SCF plus IL-2 stimulation**

The MAPK signal transduction pathway is activated in response to IL-2/15 stimulation via the IL-2/15R β and γ subunits, and in response to SCF activation of c-kit. DERL-7 cells were starved in SFM for 24 hours. After 15 minutes of stimulation with SCF, IL-2, or the SCF plus IL-2, whole-cell lysates were made from the NK-cell line DERL-7 and proteins were resolved by SDS-PAGE. Immunoblotting for phosphorylated ERK was performed. As shown in Figure 3A, ERK was phosphorylated in response to SCF or IL-2 stimulation. However, SCF plus IL-2 led to greater levels of phosphorylated ERK species. Band densitometry conducted on the Western blots indicated that differences observed were statistically significant (Figure 3A bottom panel, P < .05 for indicated pairwise comparisons).

We confirmed this finding in fresh primary human CD56bright NK cells, as seen in Figure 3B. The phosphorylation of ERK was markedly enhanced after 15 minutes of costimulation with SCF plus IL-2, compared with the effect observed in response to either SCF or IL-2 alone. Once again, densitometry revealed statistically significant differences between the intensity of the bands observed (Figure 3B bottom panel, P < .05 for indicated pairwise comparisons).

In contrast to the findings in the MAPK pathway, no significant differences were observed in activation of signaling intermediaries in other signal transduction pathways activated by c-kit or IL-2/15R. Specifically, there was no evidence for enhanced tyrosine phosphorylation of STAT3 or STAT5, or in serine phosphorylation of STAT3 as had been shown in response to SCF and G-CSF (data not shown). Similarly, the combination of SCF plus IL-2 did not alter signaling intermediates in the PI3K pathway compared with stimulation with SCF or IL-2 alone (data not shown).

**IL-2– and SCF plus IL-2–mediated proliferation are MAPK dependent**

Given the increase in phosphorylation of MAPK–signaling intermediaries, we sought to further characterize the functional correlates of this finding. The MAPK-signaling pathway has previously been shown to be critical in mediating the proliferative response of NK cells to IL-2/15. Figure 4A shows combined data from 3 independent experiments in fresh primary, human NK cells cultured in SCF, IL-2, or the combination either in DMSO or 10 μM U0126, a small molecule MEK1/2-specific inhibitor. We confirmed that NK-cell proliferation in response to IL-2 is dependent on MAPK signaling, as the presence of the MAPK inhibitor impaired the proliferative response compared with cells stimulated with IL-2 in DMSO (P < .05). MAPK inhibition also impaired proliferation in response to SCF plus IL-2 (P < .05) as well. To confirm the idea that the MAPK pathway mediates enhanced proliferation in response to SCF plus IL-2, we next tested the complementary concept, that is, whether overexpression of ERK1 and ERK2 might lead to enhanced proliferation.

Statistically significant (P = n.s.). (C) A similar phenomenon is observed in primary human NK cells that show the greatest loss of p27 expression in the top panel is in response to SCF + IL-2 compared with SCF or IL-2 alone after 18 hours. Band densitometry in the bottom panel again shown as composite mean (± SEM) from 2 independent experiments in 2 different donors confirms statistical significance of differences (for comparisons indicated by * in panel C, P < .05 for all pairwise comparisons). (D) The y-axis represents the mean percentage proliferation (± SEM) above that measured for untreated controls as measured by the MTS assay, of NK-cell line DERL-7 in response to cytokine stimulation preincubated in a selective CDK4 inhibitor (PD032991) compared with vehicle (DMSO) alone. Statistically significant differences were observed in proliferation in all conditions as a function of CDK4 inhibition (*P < .05, "P < .01, and **P < .001). Results shown are combined from 3 independent experiments.
Wild-type ERK1 and ERK2 were overexpressed in the DERL-7 NK-cell line. Although ERK1 and ERK2 overexpression did not significantly affect SCF-mediated growth, statistically significant increases in proliferative response to IL-2 and SCF plus IL-2 were observed. Combined results from 4 independent experiments (shown in Figure 4B) demonstrate that ERK1 and ERK2 overexpression led to statistically significant 55% to 79% increases in proliferative response to IL-2 and IL-2 plus SCF, respectively. These results lend further support to the idea that costimulation with SCF leads to enhanced MAPK signaling, augmenting the proliferation signal mediated through activation of the IL-2/15R in NK cells.

Costimulation with SCF plus IL-2 facilitates G1/S checkpoint progression

Activation of MAPK signaling leads to a myriad of downstream effects that may ultimately direct cellular proliferation; in particular, several regulators of the G1/S cell-cycle checkpoint have been shown to be important downstream targets of activated ERK in lymphocytes. Therefore, we hypothesized that one potential mechanism of synergy would be a facilitative effect of SCF costimulation on G1/S checkpoint progression mediated by IL-2/15R activation of MAPK signaling. After 48 hours in SFM, the NK-cell line DERL-7 is more than 95% viable by trypan blue staining under light microscopy. The cells are virtually entirely in the G0 phase of the cell cycle (Figure 5A left panel). After 24 hours of stimulation with either SCF, IL-2, or SCF plus IL-2, a proportion of each population transitioned into the cell cycle. Across 3 independently conducted experiments, after 24 hours of cytokines, an average of 16% (range, 13%-18%) of cells stimulated with SCF were beyond G1/S, 25% (range, 20%-32%) of cells stimulated with IL-2 were beyond G1/S, and 36% (range, 29%-48%) of cells stimulated with IL-2 and SCF together were beyond G1/S (P < .05). A representative result is shown in Figure 5A. Thus, the enhanced MAPK signaling seen in response to costimulation with SCF plus IL-2 leads ultimately to synergistic proliferation through facilitation of entry into the cell cycle.

Costimulation with IL-2 and SCF uniquely modulates expression of p27 in MAPK-dependent manner

Synergistic proliferation between SCF and G-CSF has been shown to result in part from modulation of p27 expression. As p27 also controls G1/S checkpoint progression, we examined p27 expression in the DERL-7 NK-cell line as well as in primary NK cells after 6-hour stimulation with SCF, IL-2, or SCF plus IL-2. In DERL-7 cells, IL-2-dependent loss of p27 expression was augmented by costimulation with SCF (Figure 5B left panel) and was found to be MAPK dependent (Figure 5B). This finding was confirmed in primary, human NK cells (Figure 5C). In both cases, densitometry analysis of the p27 bands shows significant differences between conditions for both the DERL-7 NK-cell line (Figure 5B, P < .05) and primary, human NK cells (Figure 5C, P < .05).

CDK4 is required to facilitate SCF-, IL-2-, and IL-2 plus SCF–mediated proliferation

CDK4 is another protein implicated in control of cell-cycle entry. We sought to determine whether CDK4 activity subserves NK-cell proliferation in response to SCF, IL-2, or the combination. In the NK-cell line DERL-7 (combined results from 3 independent experiments shown in Figure 5D), proliferation was significantly impaired when cells were exposed to a selective CDK4 inhibitor (PD0332991) prior to cytokine stimulation. The modest proliferation mediated by SCF alone fell 13% (P < .05), and in response to IL-2 alone by 74% (P < .05). Proliferation in response to IL-2 plus SCF fell 93% (P < .01) in cells incubated in the CDK4 inhibitor compared with those incubated in vehicle alone. These findings suggest that CDK4 is another important mediator of cell-cycle entry of NK cells in response to SCF and IL-2 or the combination.

Cyclins D2 and D3 have both been implicated in lymphocyte proliferation, thus, we also studied whether these proteins are uniquely modulated by IL-2/15 and SCF. In both our DERL-7 NK-cell line and in primary human NK cells, Western blots for cyclin D2 and D3 performed after 18 hours of exposure to IL-2, SCF, or the combination led to modest differences in protein expression that were not statistically significant by band densitometry evaluation (data not shown).

Discussion

In this report, we investigate the molecular mechanisms by which proliferation of IL-2/15–stimulated human NK cells is enhanced through costimulation with SCF. The NK cell increases its surface expression of c-kit following activation by IL-2. In the presence of SCF, the tyrosine kinase activity of c-kit is necessary to enhance the activation of signaling intermediaries in the MAPK pathway initiated by IL-2/15. These events ultimately facilitate earlier entry into the cell cycle at least in part through modulation of CDK4 and p27 expression.

We recently discovered that a CD34highCD45RA– integrin β7high population of HPCs resides in human secondary lymphoid tissue (SLT). This CD34+ cell appears to subsequently acquire c-kit (CD117) and the receptor components to bind both IL-2 and IL-15. Concomitantly, it acquires CD56 and exits the lymph node as a CD56bright NK cell, unique among circulating lymphocytes in its expression of c-kit and the high-affinity heterotrimERICIL-2Rαβγ. C-kit is lost as the NK cell completes differentiation to a CD56dimCD16+ cytolytic effector cell. The observation that c-kit is acquired during NK-cell development in SLT and is retained until it appears in the peripheral circulation points to its likely importance during NK development. The fact that the CD56bright cell demonstrates enhanced proliferation in the presence of SCF and IL-2 or IL-15 would suggest that SCF is important for maintaining NK cell homeostasis. Further support of this notion comes from elucidating a role for SCF in human CD56bright NK survival and from malignant transformation associated with activating mutations of c-kit, including NK-cell leukemia. Thus, understanding how the IL-2/15R interacts with c-kit at the molecular level not only can help us characterize normal lymphopoiesis, but also can serve as a framework for understanding leukemogenesis.

NK-cell development and proliferation are the result of a highly orchestrated process of cellular sensitivity to growth factor stimulation through expression of various but specific cognate receptors on the cell surface. Although the signal transduction pathways that emanate from many of these receptors are well characterized, how they interact with each other to direct lymphopoiesis is only now beginning to be understood. The present work provides a potential molecular mechanism by which 2 of these receptors, c-kit and the heterodimeric IL-2/15Rβγ complex, along with their cognate ligands, help to orchestrate normal NK-cell lymphopoiesis and NK-cell homeostasis in humans.
Genetic disruption studies in the mouse have clarified that it is IL-15 and its high-affinity single-chain IL-15Ra that are responsible for NK-cell development and NK-cell survival, not IL-2. Nonetheless, we have identified a heterotrimeric IL-2Rαβγ, on the surface of both the CD34dimCD45RA+ integrin β2+ population and the CD56bright NK cell, both of which express c-kit. In humans, IL-2 appears to be provided by T cells following their activation. Its restricted provision is consistent with the fact that it is not pivotal for NK-cell development. However, both NK-cell precursors and CD56bright NK cells reside in the parafollicular T cell–rich regions of SLTs where foreign proteins are processed by antigen-presenting cells to activate T cells. Thus, at the time of antigenic stimulation by either infectious agents or mutated cancer cells in the SLT microenvironment, these NK intermediaries may successfully compete for T cell–derived IL-2 that, with the abundance of SCF in normal human serum, has the potential to result in a robust and synergistic NK-cell proliferative response via the molecular mechanisms described in this report. The same can be said for the presentation of membrane-bound IL-15 by antigen- or cytokine-activated dendritic cells (DCs).

Interestingly, several the molecular pathways that have been elucidated to explain synergy when SCF is combined with IL-3, GM-CSF, G-CSF, and EPO cannot be used to explain the observed synergy in proliferation seen with SCF and IL-2/15. For example, c-kit has been shown to colocalize with the erythropoietin receptor and the GM-CSF receptor. Although in one experiment we found colocalization of c-kit with the IL-2/15Rβ, we could not reproducibly demonstrate this relationship between c-kit and other elements of the IL-2/15R.

We do show that signaling intermediaries within the MAPK pathway are activated both by SCF and IL-2/15 and selectively enhanced when these 2 cytokines are delivered in combination. This in turn facilitates earlier entry into the cell cycle, resulting at least in part via down-regulation of the p27 tumor suppressor gene, also mediated via the MAPK pathway. That the presence of functional CDK4 activity is required for SCF, IL-2, and particularly SCF plus IL-2–mediated proliferation also supports the idea that these cytokine-initiated signaling pathways culminate in modulating the G1/S checkpoint in the proliferation response in NK cells. These data are consistent with and extend prior research suggesting MAPK signaling targets mediators of G1/S progression. To our knowledge, this mechanism of cytokine signaling synergy between SCF and IL-2/15 has not been previously described in primary human or mouse cells.

The other combination of cytokines likely more relevant to NK-cell development is IL-15 and SCF. IL-15 is critical for commitment of CD34 HPCs toward the NK-cell lineage and it would seem plausible that SCF could serve to enhance the proliferative response during differentiation to meet the normal demands of maintaining NK homeostasis. Although the length of survival for a human NK-cell has not yet been accurately determined, at any one time there are likely in excess of 2 billion NK cells circulating in a healthy adult. We have previously shown that although IL-15 alone can differentiate NK cells from CD34+ HPCs, SCF can greatly augment the number of cells resulting from this process, largely from 2 distinct processes: first, the up-regulation of the IL-15Rβ and γ chains on CD34+ HPCs, thereby increasing the frequency of NK precursors among CD34+ HPCs, and second, via the mechanism described in this report for enhanced cellular proliferation for the CD56bright NK cell. As noted earlier, the c-kit and SCF axis likely also contributes to NK-cell homeostasis as by itself, it can prolong CD56bright NK cell survival in SFM, a property shared by IL-2/15 but not by other cytokines or growth factors. In data not shown, we have noted that for NK cells stimulated with IL-15, the MAPK inhibitor UO126 abrogates survival, whereas for NK cells stimulated with SCF, the c-kit kinase inhibitor AG1296 abrogates survival. However, for NK cells stimulated with SCF plus IL-15, neither the MAPK inhibitor alone nor the kit kinase inhibitor alone significantly attenuates NK-cell survival compared with vehicle-treated controls. Thus, these 2 ligands appear to use 2 distinct pathways to promote NK-cell survival.

It is interesting that SCF can up-regulate components of the IL-2/15R on the surface of CD34+ cells and on the surface of IL-2–dependent intraepithelial lymphocytes but not on the surface of CD56bright NK cells. In contrast, incubation of the CD56bright NK cell in the presence of IL-2 does up-regulate c-kit expression on these cells. One can speculate that when delivering a survival signal, the ubiquitous SCF is sufficient by itself, yet when delivering a proliferative signal, the activation-induced provision of IL-2 or IL-15 also up-regulates c-kit to augment the human immune response in protecting the host.

Taken together, we provide what we believe are novel mechanistic insights into the observed proliferative synergy when CD56bright NK cells receive signals from both SCF and IL-2 or IL-15. We believe that elucidation of such mechanisms will provide a foundation for understanding lymphocyte development and homeostasis, as well as insights into the potential causes of malignant transformation and potential targets for nontoxic therapies.

Acknowledgments
This study was supported by National Cancer Institute (NCI)/National Institutes of Health (NIH, Bethesda, MD) P01 CA95426-01 (M.A.C.), NCI/NIH R01 CA68458-08 (M.A.C.), American Society of Clinical Oncology Young Investigator Award (D.M.B. and M.A.C.), and NCI/NIH T32 CA09338-26 (D.M.B. and M.A.C.).

Authorship
Contribution: D.M.B. wrote the paper, and designed and conducted research; J.Y. and B.B. designed and conducted research; M.W. conducted research; A.G.F. designed experiments; A.K.F. conducted statistical analyses; R.T., D.P., and R.B. provided reagents and designed research; M.A.C. supervised the project in its entirety and wrote the paper; and all authors approved the final version of the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Stem cell factor and interleukin-2/15 combine to enhance MAPK-mediated proliferation of human natural killer cells

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