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

Don M. Benson Jr,1 Jianhua Yu,2,6 Brian Becknell,3 Min Wei,2 Aharon G. Freud,3
Amy K. Ferketich,4 Rossana Trotta,2,6 Danilo Perrotti,2,6 Roger Briesewitz,5,6
and Michael A. Caligiuri1,3,6

From the 1Division of Hematology/Oncology, 2Department of Molecular Virology, Immunology and Medical Genetics, 3Medical Scientist Program, Integrated Biomedical Graduate Program, College of Medicine, 4Division of Biostatistics, College of Public Health, 5Department of Pharmacology, and the 6Comprehensive Cancer Center, The Ohio State University, Columbus, OH, 43210.

Address correspondence to: Michael A. Caligiuri, MD, The Comprehensive Cancer Center, The James Cancer Hospital and Solove Research Institute, The Ohio State University, 300 W 10th Ave, Columbus, Ohio, 43210-1240. email: Michael.caligiuri@osumc.edu

Running Title: Mechanisms of proliferative synergy between SCF and IL-2/15
Abstract

Stem cell factor (SCF) promotes synergistic cellular proliferation in combination with a number of growth factors, and appears important for normal natural killer (NK) cell development. CD34(+) hematopoietic precursor cells (HPC) require interleukin (IL)-15 for differentiation into human NK cells, and this effect can be mimicked by IL-2. Culture of CD34(+) HPC or some primary human NK cells in IL-2/15 and SCF results in enhanced growth compared with either cytokine alone. The molecular mechanisms responsible for this are unknown and were investigated in the present work. Activation of NK cells by IL-2/15 increases expression of c-kit whose kinase activity is required for synergy with IL-2/15 signaling. Mitogen activated protein kinase (MAPK) signaling intermediaries that are activated both by SCF and IL-2/15 are enhanced in combination to facilitate earlier cell cycle entry. The effect results at least in part via enhanced MAPK-mediated modulation of p27 and CDK4. Collectively the data reveal a novel mechanism by which SCF enhances cellular proliferation in combination with IL-2/15 in primary human NK cells.
Introduction

Stem cell factor (SCF, also known as c-kit ligand, steel factor, and mast cell growth factor), synergizes with hematopoietic growth factors like erythropoietin, GM-CSF, G-CSF, to promote lineage-specific, hematopoietic precursor cell growth. IL-15 mediates differentiation of CD34^dimCD45RA(+) integrin β7^bright hematopoietic precursor cells (HPC) into human natural killer (NK) cells. This can be mimicked by IL-2 signaling through the same βγ receptor complex. 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. In combination with IL-2/15, SCF enhances growth of CD34^dimCD45RA(+) integrin β7^bright HPC during NK cell differentiation and enhances the growth of CD56^bright c-kit(+) NK cells, yet by itself, SCF does not promote significant human NK cell proliferation. The relevance of c-kit and SCF to NK cell development and expansion are illustrated by genetic disruption of these molecules in mice which reveal a quantitative NK cell deficiency. Whereas the molecular mechanisms describing the synergy between SCF and EPO, G-CSF, and GM-CSF in promoting cell growth have been characterized, similar studies examining SCF and IL-2/15 have not been reported.

Three major mitogen activated protein kinase (MAPK) pathways have been identified and when activated, result in kinase-mediated phosphorylation of p42/44, also known as extracellular regulated kinases (ERK), stress-activated protein kinase-2/p38, and Jun kinases (JNK). Signaling through the MAPK pathway, particularly through activation of ERK, contributes critically to NK cell proliferation and NK cell development. MAPK-ERK signaling is stimulated through the binding of extracellular
growth factors like SCF and IL-2/15 to their cognate transmembrane receptors. The phosphorylated intracytoplasmic tail of the receptor in turn recruits Grb2 that binds guanine exchange factor SOS at the membrane. SOS then interacts with H-ras that in turn leads to its activation via GTP loading followed by the sequential recruitment and activation of Raf, 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. Activated ERK then dimerizes and translocates to the nucleus and induces gene expression required for cell proliferation, development, and survival.

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.
Materials and Methods

Antibodies and Inhibitors Mabs specific to phospho-MEK (#9121), phospho-Erk1/2 (#9101), p27 (#2552) and the MEK1/2 specific kinase inhibitor UO126 (#9903) were purchased from Cell Signaling Technology (Beverly, MA). The c-kit tyrosine kinase inhibitor AG1296 (#658551) was obtained from Calbiochem (San Diego, CA). The CDK4 specific inhibitor, PD0332991, was obtained from Pfizer Pharmaceuticals (New York, NY). Anti-actin goat polyclonal antibody was purchased from Sigma (St Louis, MO). Anti-CD117-PE as well as isotype IgG-PE control were purchased from BD Biosciences (San Jose, CA). Antibodies to Cyclin D2 (#2924) and Cyclin D3 (#2936) were purchased from Cell Signaling Technology. K-27 was a kind gift of Yosef Yarden (Weizmann Institute, Rehovot, Israel).

Cell line and primary NK cell preparation The IL-2 dependent CD3(-)CD56(+) NK cell line DERL-7\textsuperscript{12} (a generous gift from R. Di Noto and CEINGE Biotechnologie, 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 CD56\textsuperscript{bright} subset.\textsuperscript{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 individuals (American Red Cross, Columbus, OH) and maintained
as previously described. Briefly, NK cells were processed using the RosetteSep NK cell antibody cocktail (StemCell Technologies, Vancouver, BC), followed by Ficoll-Hypaque density centrifugation. Enriched NK cells were then expanded for one week in RPMI 1640 (Invitrogen), supplemented with 10% heat-inactivated human AB serum (ICN Biomedicals, Irvine, CA), antibiotics, 10 mM HEPES, 100 μM nonessential amino acids, 1 mM Na pyruvate, and 50 μM 2-βME (Sigma) plus 150 IU/mL IL-2 (Hoffman-LaRoche, Nutley, NJ), and 250 ng/mL SCF (Amgen, Thousand Oaks, CA). Experiments reported herein were conducted after this expansion period and following a 12 h overnight incubation in serum free medium at 37°C without any cytokines or growth factors.

**Cell proliferation assays** For 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) proliferation assays (CellTiter 96 AQuescent One Solution Cell Proliferation Assay, Promega, Madison, WI), cells were washed in RPMI and resuspended in serum-free RPMI at 10^7 cells / mL without cytokines for 48-h. Twenty thousand cells were seeded in each well of a 96-well flat bottom plate. As appropriate, cells were treated with AG1296 (5uM), UO126 (10uM), or PD0332991 (500 nM) for 2 hours prior to addition of cytokines. Cytokines were added where appropriate at the following concentrations: SCF 100 ng/mL (NK cell line DERL-7) or 250 ng/mL (human primary NK cells) and / or IL-2 150 IU / mL or IL-15 20 ng/mL (Amgen). After exposure to cytokines for 24 or 48-h, 20uL of MTS reagent was added to each well. Two hours later, optical density of cells at 495 nm was recorded and proliferation was assessed per manufacturer’s instructions for cells in each condition.
as expressed as percent growth in relation to untreated controls being 0%. Cells were also counted by light microscopy using a hemocytometer by trypan blue exclusion.

**Immunoblotting** Cells were pelleted and lysed at 10^7/mL with ice-cold lysis buffer (1% Nonidet P-40, 10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] pH 7.5, 0.15 to 0.5 M NaCl, 10% glycerol supplemented with protease and phosphatase inhibitors, 1 mM phenylmethylsulfonylfluoride [PMSF], 1 mM Na_3 VO_4, 50 mM NaF, 10 mM β-glycerol-phosphate, 1 mM EDTA), and a protease inhibitor cocktail tablet from Roche Applied Science (Indianapolis, IN) as described previously. Lysates were cleared by centrifugation for 20-min at 4°C at 14,000 rpm. Protein concentration of lysates was derived by optical density of solutions at 595 nm using Bio-Rad protein assay (BIO-RAD Laboratories, Hercules, CA) per the manufacturer’s instructions.

Western blotting was performed according to previously published protocols. Briefly, proteins were analyzed by 4% to 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (BIO-RAD Laboratories) using reducing conditions. Samples were transferred to nitrocellulose membranes (Protran BA85, Whatman, Inc., Sanford, ME) and mAb-reactive proteins were detected with horseradish peroxidase–labeled sheep anti–rabbit, –mouse, and/or –goat immunoglobulin (Ig) sera and enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

**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 two washes, cells were resuspended in ice cold ethanol and kept at -20°C until analysis. Propidium iodide staining solution (50μg/mL)
was added with RNase A stock solution (20ug/mL). Data were collected using DNA QC software (BD Biosciences, San Jose, CA) according to manufacturer’s protocol, running on a FACS Caliber Flow Cytometer (BD Biosciences, San Jose, CA) and were analyzed using Cell Quest software (BD Biosciences).

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

*Statistical Considerations*

For intergroup comparisons between cytokine-stimulated conditions in proliferation assays, one-way ANOVA was used to assess group effects with subsequent multiple comparisons by Tukey’s 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 \textit{a priori} specified comparison was examined, a Bonferroni correction (alpha for each test was set to 0.016) was employed. 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

Co-stimulation with SCF and IL-2/15 leads to synergistic proliferation of NK cells. We first confirmed that the CD56<sup>bright</sup> NK cell line DERL-7 displays a modest amount of proliferative synergy in response to culture with SCF and IL-2 (Fig 1A) or with SCF and IL-15 (Fig 1B). In all circumstances, proliferation is measured percent above that of untreated controls. Cells cultured in serum free medium (SFM) with SCF (100ng/mL) alone for 48-h induced 96 +/- 2.2% (SEM) growth, whereas IL-2 (150IU/mL) induced 206 +/- 5.2% growth, and the combination resulted in 360 +/- 8.6% growth. As shown in Fig 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-h, SCF (100ng/mL) yielded 29 +/- 4.5% growth, IL-15 (20ng/mL) produced 400 +/- 14% growth and the combination led to 471 +/- 7.7% growth. Fig 1C confirms our previous observation of clear synergistic proliferation in fresh primary resting human CD56<sup>bright</sup> NK cells using SFM, where SCF led to 8 +/- 2.1% growth, IL-2 produced 95 +/- 7% growth, and SCF + IL-2 led to 150 +/- 6% proliferation. Differences shown in Fig 1A, B, and C are combined data from at least three independent experiments in each circumstance and all pair wise comparisons referenced were statistically significant (p < 0.01). In addition, the synergistic proliferation seen in primary human NK cells also occurs when CD34<sup>dim</sup>CD45RA+ HPC are stimulated with SCF + IL-15 compared with either cytokine alone (p < 0.05, data not shown).

IL-2 stimulation upregulates 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.\textsuperscript{1,2} DERL-7 cells starved for 48-h in SFM were then stimulated with SCF, IL-2, or SCF + IL-2 and surface density expression of c-kit as well as the IL-2/15 β and γ subunits was assessed by flow cytometry. IL-2 stimulation led to a statistically significant upregulation of CD117 (c-kit) expression (Fig 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, \textit{p} < 0.001). 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 nor the NK cell line DERL-7 (data not shown).

\textit{The tyrosine kinase activity of c-kit is required for SCF-induced proliferation as well as proliferative synergy.} We hypothesized that upregulation 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 or not a functional consequence of upregulated CD117 expression might occur. The DERL-7 NK cell line was starved 48-h in SFM and then incubated for 2-h in DMSO (vehicle) or AG1296 (5 uM), a tyrosine kinase inhibitor specific for c-kit.\textsuperscript{2} By Western blotting, DERL-7 cells incubated in SCF have increased levels of phospho-ERK compared to 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 the presence of the c-kit inhibitor, AG1296. DERL-7 cells incubated in SCF+IL-2 have increased phospho-ERK levels which are greater than the phospho-ERK levels seen in the presence of IL-2 or SCF alone. DERL-7 cells incubated in IL-2+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 + IL-2 for 48-h, followed by a 2-h measurement of proliferation. Combined data from three independent experiments in the NK cell line DERL-7 is shown in Fig 2B demonstrating that inhibition of c-kit tyrosine kinase activity abolishes the sum total contribution to the modest proliferative synergy in the SCF + IL-2 condition. Culture in SCF alone led to 93 +/- 5.2% growth in vehicle (DMSO) versus unstimulated cells in SFM. However, in the presence of AG1296, the proliferative response was virtually abolished, measuring only 0.5 +/- 0.7% of untreated controls (p < 0.001). Culture in IL-2 alone led to 234 +/- 6.2% growth that was partially suppressed to 163 +/- 3.5% in the presence of AG1296 (p < 0.05).

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 upregulates surface expression of available c-kit (as shown previously in Fig 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-2/15 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.2 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.\textsuperscript{2}

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 co-localize with c-kit to facilitate proliferation.\textsuperscript{1,2} In fact, using a co-immunoprecipitation / Western blot technique, we observed reciprocal colocalization between c-kit and the IL-2/15R\(\beta\) chain (data not shown), but we could not demonstrate such co-localization between IL-2/15\(\gamma_c\) 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 + IL-2 in DMSO was 357 +/- 7\% above untreated controls, while the addition of AG1296 allowed IL-2 + SCF mediated proliferation to increase by only 185 +/- 3.2\% above untreated controls, \((p < 0.01)\). 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.

\textit{MAPK signal transduction is uniquely modulated by SCF + IL-2 stimulation.}

The MAPK signal transduction pathway is activated in response to IL-2/15 stimulation via the IL-2/15R \(\beta\) and \(\gamma\) subunits\textsuperscript{16}, and in response to SCF activation of c-kit.\textsuperscript{2} DERL-7 cells were starved in SFM for 24-h. After 15-min of stimulation with SCF, IL-2 or the SCF + 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 Fig 3A, ERK was phosphorylated in response to SCF or IL-2 stimulation. However, SCF + IL-2 led to greater levels of phosphorylated ERK species. Band
densitometry conducted on the Western blots indicated that differences observed were statistically significant (Fig 3A bottom panel, \( p < 0.05 \) for indicated pair-wise comparisons).

We confirmed this finding in fresh primary human CD56\(^{\text{bright}}\) NK cells, as seen in Fig 3B. The phosphorylation of ERK was markedly enhanced after 15-min of co-stimulation with SCF + 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 (Fig 3B bottom panel, \( p < 0.05 \) for indicated pair-wise 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\(^3\) (data not shown). Similarly, the combination of SCF + IL-2 did not alter signaling intermediates in the PI3K pathway when compared to stimulation with SCF or IL-2 alone (data not shown).

**IL-2- and SCF+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.\(^{10}\) Fig 4A shows combined data from three independent experiments in fresh primary, human NK cells cultured in SCF, IL-2 or the combination either in DMSO or 10uM of UO126, 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 ($p < 0.05$). MAPK inhibition also impaired proliferation in response
to SCF + IL-2 ($p < 0.05$), as well. To confirm the idea that MAPK pathway mediates
enhanced proliferation in response to SCF + IL-2, we next tested the complementary
concept, i.e., whether over expression of ERK1 and ERK2 might lead to enhanced
proliferation.

Wild-type ERK1 and ERK2 were over expressed in the DERL-7 NK cell line. Although ERK1 and ERK2 over expression did not significantly affect SCF mediated
growth, statistically significant increases in proliferative response to IL-2 and SCF + IL-2
were observed. Combined results from four independent experiments (shown in Fig 4B)
demonstrate that ERK1 and ERK2 over expression led to statistically significant 55 –
79% increases in proliferative response to IL-2 and IL-2 + SCF. These results lend
further support to the idea that co-stimulation with SCF leads to enhanced MAPK
signaling, augmenting the proliferation signal mediated through activation of the IL-
2/15R in NK cells.

Co-stimulation with SCF + 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, a number of 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 co-stimulation on G1/S checkpoint
progression mediated by IL-2/15R activation of MAPK signaling. After 48-h in SFM,
the NK cell line DERL-7 is > 95% viable by trypan blue staining under light microscopy. The cells are virtually entirely in the G0 phase of the cell cycle (left panel, Fig 5A). After 24-h of stimulation with either SCF, IL-2, or SCF + IL-2, a proportion of each population transitioned into the cell cycle. Across three independently conducted experiments, after 24-h of cytokines, an average of 16% (range 13-18%) of cells stimulated with SCF were beyond G1/S, 25% (20-32%) of cells stimulated with IL-2 were beyond G1/S, and 36% (29 – 48%) of cells stimulated with IL-2 and SCF together were beyond G1/S (p < 0.05). A representative result is shown in Fig 5A. Thus, the enhanced MAPK signaling seen in response to co-stimulation with SCF + IL-2 leads ultimately to synergistic proliferation through facilitation of entry into the cell cycle.

Co-stimulation 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-h stimulation with SCF, IL-2 or SCF+IL-2. In DERL-7 cells, IL-2 dependent loss of p27 expression was augmented by co-stimulation with SCF (left panel, Fig 5B) and was found to be MAPK-dependent (Fig 5B). This finding was confirmed in primary, human NK cells (Fig 5C). In both cases, densitometry analysis of the p27 bands shows significant differences between conditions for both the DERL-7 NK cell line (Fig 5B, p < 0.05) and primary, human NK cells (Fig 5C, p < 0.05).

CDK4 is required to facilitate SCF, IL-2 and IL-2+SCF mediated proliferation. CDK4 is another protein implicated in control of cell cycle entry. We sought to determine whether or not 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 three independent experiments shown in Fig 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% ($p < 0.05$), and in response to IL-2 alone by 74% ($p < 0.05$). Proliferation in response to IL-2 + SCF fell 93% ($p < 0.01$) 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.

Cyclin D2 and D3 have both been implicated in lymphocyte proliferation, thus, we also studied whether or not 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-h 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 co-stimulation 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 CD34dimCD45RA(+)integrin β7bright population of HPC 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 heterotrimeric IL-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 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 cannot only help us characterize normal lymphopoiesis, but can also 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 two 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-15Rα 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 β7bright population and the CD56bright NK cell, both of which express c-kit. In humans, IL-2 appears to only 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 SLT where foreign proteins are processed by antigen presenting cells in order 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 which, with the abundance of SCF in normal human serum, have 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 DC.29

Interestingly, a number of the molecular pathways that have been elucidated to explain synergy when SCF is combined with IL-3,18 GM-CSF,2 G-CSF,3 and erythropoietin1 cannot be utilized to explain the observed synergy in proliferation seen with SCF and IL-2/15. For example, c-kit has been shown to co-localize with the erythropoietin receptor1 and the GM-CSF receptor.2 Although in one experiment we found co-localization 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 two cytokines are delivered in combination. This in turn facilitates earlier entry into the cell cycle, resulting at least in part via downregulation 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 +IL-2 mediated proliferation also supports the idea 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.9 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 HPC toward the NK cell
lineage and it would seem plausible that SCF could serve to enhance the proliferative response during differentiation in order to meet the normal demands of maintaining NK homeostasis. While 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 two billion NK cells circulating in a normal adult. We have previously shown that while IL-15 alone can differentiate NK cells from CD34(+) HPC, SCF can greatly augment the number of cells resulting from this process, largely from two distinct processes: first, the upregulation of the IL-15Rβ and γ chains on CD34(+) HPC, thereby increasing the frequency of NK precursors among CD34(+) HPC, 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 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, while for NK cells stimulated with SCF, the c-kit kinase inhibitor AG1296 abrogates survival. However, for NK cells stimulated with SCF + IL-15, neither the MAPK inhibitor alone nor the kit kinase inhibitor alone significantly attenuate NK cell survival compared to vehicle-treated controls. Thus, these two ligands appear to utilize two distinct pathways to promote NK cell survival.

It is interesting that SCF can upregulate 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 upregulate 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 upregulates 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 CD56<sup>bright</sup> 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 non-toxic therapies.
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Author Contributions

DMB wrote the paper, designed and conducted research. JY designed and conducted research, BB designed and conducted research, MW conducted research, AGF designed experiments, AKF conducted statistical analyses, RT provided reagents and designed research, DP provided reagents and designed research, RB provided reagents and designed research, MAC supervised the project in its entirety and wrote the paper, all authors approved the final version of the manuscript.

Conflicts of Interest Disclosure

No authors have conflicts of interest to disclose regarding commercial relationships or any other financial relationships.
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Figure legends

Figure 1. SCF and IL-2/15 lead to modest synergistic proliferation of NK cells. For Figs. 1A-C, the Y axis represents the mean percent proliferation (+/- SEM) above that measured for untreated controls as measured by the MTS assay. Fig 1A: Proliferation of the NK cell line DERL-7 in response to stimulation with SCF, IL-2 or SCF + IL-2. Cells were starved in SFM for 48-h, washed and stimulated in triplicate wells with SCF (100 ng/mL), IL-2 (150 IU/mL) or SCF + IL-2 for 48-h. Fig. 1B: IL-15 (20ng/mL) is substituted for IL-2 and leads to similar findings as seen in Fig. 1A. Fig. 1C: Modest synergistic proliferation of primary, human CD56^{bright} 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.6 All pair-wise comparisons are statistically significant between treatment groups (p < 0.05) in Figs 1A, B, and C, and each figure shows combined data from at least three independent experiments.

Figure 2. CD117 expression is increased by IL-2 stimulation; c-kit tyrosine kinase activity is required for synergy. Fig 2A: From a representative experiment of five, IL-2 stimulation upregulates c-kit expression in the NK cell line DERL-7 as opposed to unstimulated cells. Fig 2B: Starved DERL-7 cells, pre-treated for 2-h with AG1296, a c-kit specific tyrosine kinase inhibitor were stimulated with SCF, IL-2 or SCF + IL-2. Proliferation was measured using an MTS reagent, measuring OD at 490nm. Data shown are combined from three independent experiments, demonstrating mean percent proliferation (+/- SEM). AG1296 virtually abolished any SCF-dependent proliferation (left bars, p < 0.01) and a modest impact was seen on IL-2 mediated proliferation (center
bars, \( p < 0.01 \). The main finding (comparison indicated by “*” in Fig 2B) is that the sum total contribution of SCF to synergistic proliferation was abrogated in the presence of AG1296 \( (p < 0.01) \), as proliferation in response to IL-2 was identical to that of SCF + IL-2 \( (p = n/s) \).

**Figure 3. Phosphorylated-ERK is uniquely increased in response to SCF + IL-2 as opposed to SCF or IL-2 alone.** Fig 3A: The upper 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 reveals statistically significant differences in composite mean (+/- SEM) phospho-ERK levels (relevant pair-wise comparisons indicated by “*” in Fig 3A, \( p < 0.05 \)) from three independent experiments. Fig 3B: 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 four independent experiments shown are also statistically significant (relevant pair-wise comparisons indicated by “*” in Fig 3B, \( p < 0.05 \)).

**Figure 4. IL-2 mediated NK cell proliferation as well as SCF + IL-2 proliferative synergy are MAPK-dependent.** For Figs. 4A-B, the Y axis represents the mean percent proliferation (+/- SEM) above that measured for untreated controls as measured by the MTS assay. Fig 4A: Primary, human NK cells pretreated with either UO126 (10uM) or
DMSO (vehicle) were cultured in SCF, IL-2 or SCF + IL-2. Combined data from three 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 Fig4A, $p < 0.05$ for each comparison shown).

Fig 4B: Over expression of ERK1 and ERK2 in the NK cell line DERL-7 yields complementary results. Over expression of ERK1 (light gray bar) and ERK2 (dark gray bar) led to statistically significant increases in proliferation (+/- SEM) in response to IL-2 and SCF + IL-2 (comparisons indicated by “*” respectively in Fig 4B, $p < 0.05$ for all indicated comparisons) compared with cells treated with vector alone (white bars).

**Figure 5.** MAPK-mediated synergistic proliferation is mediated in part by unique modulation of the G1/S cell cycle checkpoint. Fig 5A: The NK cell line DERL-7 is > 95% viable by trypan blue staining and is entirely in G0 after 48-h in SFM (left panel). After 24-h 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 ($p < 0.05$). Statistically significant results are representative of 3 independent experiments. Fig 5B: Starved DERL-7 cells were stimulated with SCF, IL-2 or SCF + IL-2 for 18-h in the presence of DMSO (carrier) or an inhibitor of MAPK. Top: Immunoblotting for p27 in the left upper 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 lower panel shows actin as a loading control. Immunoblotting for p27 in the right upper panel reveals that, in the presence of the MAPK inhibitor UO126, there is no change in p27 expression. Lower left: the bar graph summarizes band densitometry data.
shown as the composite mean (+/- SEM) from three 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 Fig 5B, \( p < 0.05 \) for all pair-wise comparisons). Lower right: the bar graph summarizes band densitometry data shown as the composite mean (+/- SEM) from three independent immunoblotting experiments such as that shown on the top right in the presence of the MAPK inhibitor UO126. It confirms that these differences in p27 expression are not statistically significant (\( p = n/s \)). Fig 5C: A similar phenomenon is observed in primary human NK cells that show the greatest loss of p27 expression in the upper panel is in response to SCF + IL-2 compared with SCF or IL-2 alone after 18-h. Band densitometry in the lower panel again shown as composite mean (+/- SEM) from two independent experiments in two different donors confirms statistical significance of differences (for comparisons indicated by “**” in Fig 5C, \( p < 0.05 \) for all pair-wise comparisons). Fig 5D. The Y axis represents the mean percent 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 pre-incubated in a selective CDK4 inhibitor (PD0332991) compared with vehicle (DMSO) alone. Statistically significant differences were observed in proliferation in all conditions as a function of CDK4 inhibition (* represents \( p < 0.05 \), ** represents \( p < 0.01 \), *** represents \( p < 0.001 \)). Results shown are combined from three independent experiments.
Fig 2A

- Resting cells
- IL-2-stimulated cells
- Isotype

COUNTS vs. C-KIT EXPRESSION
Figure 2B: Percent proliferation above untreated controls for SCF, IL-2, and SCF+IL-2 treatments. The graph shows a significant increase in proliferation with SCF+IL-2 compared to SCF and IL-2 treatments.
Fig 3A

UNSTIM SCF IL-2 SCF+IL-2

Phospho-ERK 1/2

Actin

Phospho-ERK protein level (arbitrary densitometric units)

UNSTIM SCF IL-2 SCF+IL-2

*
Fig 3B

UNSTIM  SCF  IL-2  SCF+IL-2

Phospho-ERK 1/2

Actin

Phospho-ERK protein level (arbitrary densitometric units)

UNSTIM  SCF  IL-2  SCF+IL-2

*
Fig 4A

Percent proliferation above untreated controls

- SCF
- IL-2
- SCF+IL-2

DMSO
MAPK inhibitor

* Indicates statistical significance.
Fig 4B

Percent proliferation above untreated controls

* Vector
- ERK1
- ERK2

SCF
IL-2
SCF+IL-2
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The graph shows the p27 protein level (arbitrary densitometric units) for different treatments:

- **Fig 5B**
Fig 5C

**UNSTIM**  **SCF**  **IL-2**  **SCF+IL-2**

**p27**

![Image of Western Blot](image)

**Actin**

![Graph showing p27 protein level](graph)

p27 protein level (arbitrary densitometric units)

- UNSTIM
- SCF
- IL-2
- SCF+IL-2

*Significant difference*
Fig 5D

Percent proliferation above untreated controls

DMSO
CDK4 inhibitor

SCF
IL-2
SCF+IL-2

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

Don M Benson, Jr, Jianhua Yu, Brian Becknell, Min Wei, Aharon G Freud, Amy K Ferketich, Rossana Trotta, Danilo Perrotti, Roger Briesewitz and Michael A Caligiuri