Chapter 2

Critical roles for phosphatidylinositol-3 kinase and mitogen activated kinase kinase in cytokine-induced neutrophil differentiation

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Abstract

The production of mature blood cells, termed hematopoiesis, is a carefully orchestrated series of events involving self-renewal and differentiation of primitive pluripotent stem cells. Dysregulation of this complex process can result in severe clinical conditions ranging from aplasia of the bone marrow to leukemia. Differentiation is mediated by processes that are initiated by specific cytokine receptors, as well as processes that are intrinsically present in stem cells. To investigate the role of cytokine-activated signaling pathways in myeloid differentiation, we developed an ex-vivo maturation model. In this system, human umbilical cord blood CD34+ progenitor cells can be differentiated to mature neutrophils by addition of granulocyte-colony stimulating factor (G-CSF). First, we compared the effects of a combined cytokine-treatment utilizing interleukin (IL)-3/G-CSF, versus only G-CSF, on proliferation and differentiation. Maturation in the presence of IL-3 resulted in enhanced proliferation compared to G-CSF treatment alone. However, G-CSF induced differentiation in the absence of IL-3 resulted in a greater number of terminally differentiated neutrophils, as measured by lactoferrin- and CD11b expression, morphology, and capacity to produce reactive oxygen species (ROS). We then investigated the role of phosphatidylinositol 3-kinase (PI3K) and mitogen activated protein kinase kinase (MEK) in G-CSF induced CD34+ cell maturation using the specific pharmacological inhibitors LY294002 and PD98059 respectively. Inhibition of PI3K induced cell cycle arrest, but interestingly also resulted in impaired differentiation as was apparent by a reduction of lactoferrin positive cells and histological staining. In contrast, inhibition of MEK resulted in reduced proliferation, without significantly effecting G-CSF induced differentiation. These results demonstrate critical roles for both PI3K and MEK in the cytokine induced maturation of human CD34+ progenitor cells towards neutrophils. Furthermore, our data suggest that cell cycle arrest per se does not result in induction of an intrinsic neutrophil differentiation program.
Introduction

Hematopoiesis is a complex and carefully orchestrated series of events involving self-renewal and differentiation of primitive pluripotent stem cells. Dysregulation of hematopoietic differentiation can result in the development of a variety of pathological conditions ranging from aplasia of the bone marrow to aberrant differentiation of myeloid progenitors in diseases such as myelodysplastic syndromes and leukemia. The molecular mechanisms underlying how hematopoietic cytokines exert lineage-dominant effects on the differentiation process in vivo are not completely understood. The “inductive model” postulates that specific extracellular signals force stem cells to commit to a particular hematopoietic lineage. This model requires that the intracellular signals generated by cytokine-stimulation are specific and non-interchangeable. However, other studies have reached alternative conclusions favoring a “stochastic model”. This predicts that hematopoietic stem cell fate decisions are independent of extracellular signals and the role of cytokines is to provide non-specific survival and proliferation signals. That is, cytokines simply permit the survival, proliferation and development of intrinsically committed cells.

The effects of cytokine-stimulation are mediated by cell surface receptors belonging to the cytokine receptor superfamily, which signal by activating JAK tyrosine kinases. Interaction of cytokines with their cognate receptors expressed on hematopoietic progenitor cells, results in activation of a plethora of intracellular signaling pathways that are thought to control hematopoiesis by regulation of transcription. Activation of members of the STAT transcription factor family by multiple cytokines is thought to play a critical role in the control of hematopoietic cell growth and differentiation (reviewed in ). For example, using a mouse myeloid cell line, LGM-1, exogenously expressing the G-CSFR, STAT3 has been shown critical for neutrophil differentiation. Addition of G-CSF to these cells resulted in a block in proliferation, down-regulation of c-myc, and differentiation into cells with lobed nuclei. These changes were blocked by introduction of a dominant-negative STAT3 mutant. Several additional intracellular signaling molecules are activated by multiple cytokines, including phosphatidylinositol-3 kinase (PI3K) and Mitogen Activated Protein Kinase Kinase (MAPKK or MEK). Both signaling molecules pathways have been shown to be induced by a variety of hematopoietic cytokines including IL-3, GM-CSF and G-CSF.

PI3K has been shown to be involved in growth factor dependent survival and proliferation of a variety of cell lines. PI3K knockout mice lacking the p110α catalytic subunit die around embryonic day 9.5, which corresponds with a failure of the embryonic cells to proliferate. One mechanism for this is suggested by studies demonstrating that PI3K is involved in the regulation of the cell cycle through transcriptional induction of cyclin D. Additionally, the stabilization of cyclin D protein has been suggested to be PI3K-dependent. Furthermore, PI3K appears to play a role in down-regulation of the cyclin-dependent kinase inhibitors (CDKIs), such as p27KIP1. This is thought to be mediated, at least in part, via inactivation of the members of the Forkhead family of transcription factors. PI3K has also been linked with survival signals. Transgenic mice expressing an active form of PI3K have increased numbers of T lymphocytes exhibiting reduced apoptosis, and develop lymphoproliferative disorders.

Cytokine receptor activation also results in activation of the small GTPase Ras, which in turn can activate the protein kinase Raf. Raf activates the dual-specific MAPK Kinase (MEK), which then can phosphorylate Extracellular Regulated Kinase (ERK). Ras plays a critical role in regulating cell cycle control, possibly through down regulation of p27KIP1 and the up regulation of cyclin D1 expression. A direct role for MEK-ERK in cell cycle control has been proposed since treatment of cells with a pharmacological inhibitor of MEK (PD98059) or transfection of dominant-negative ERK correlates with decreased cyclin D1 expression.

The function of PI3K- and MEK-mediated signaling in the processes driving hematopoiesis remains unclear. In this study we developed an ex-vivo model system based on the cytokine induced differentiation of human umbilical cord blood stem cells towards neutrophils. Cytokines and colony stimulating factors including FLT-3 ligand, Stem Cell Factor (SCF), Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF), Interleukin-3 (IL-3) and Granulocyte-Colony Stimulating Factor (G-CSF) have been demonstrated to be important for granulocyte maturation. G-CSF has been demonstrated to play a critical role, as is exemplified by the neutropenia found in G-CSF deficient mice. Here we investigate a role for PI3K and MEK in the cytokine-induced proliferation and differentiation of primary human CD34+ progenitor cells towards neutrophils. We have compared maturation of isolated primary umbilical cord blood CD34+ stem cells in the presence of either a combination of IL-3 and G-CSF, or G-CSF alone. Differentiation in the presence of IL-3 increased the expansion of cells but reduced the number of
mature, functional neutrophils compared to G-CSF alone. Inhibition of PI3K during G-CSF induced maturation of CD34+ stem cells resulted in a block in proliferation and furthermore the capacity of these progenitor cells to differentiate was also abrogated. In contrast, addition of the MEK inhibitor PD98059 during the maturation process resulted in decreased proliferation, but no effect on differentiation. These data delineate critical roles for PI3K and MEK in cytokine driven differentiation and proliferation of primary hematopoietic cells towards neutrophils. Furthermore, our data suggest that cell cycle arrest per se does not result in induction of an intrinsic neutrophil differentiation program.

Materials and Methods

Reagents

Human growth factors and cytokines were used to induce differentiation. Stem Cell Factor (SCF) and FLT-3 ligand were purchased from Peprotech (Rocky Hill, NJ), IL-3 and G-CSF were obtained from Strathmann (Hamburg, Germany) and GM-CSF was purchased from Endogen (Woburn, MA). The pharmacological inhibitors PD98059 and LY294002 were purchased from Biomol (Plymouth Meeting, PA). The PE-conjugated antibody against lactoferrin was obtained from Beckman Coulter (Mijdrecht, The Netherlands) and Monoclonal antibody against CD11b (44A) was obtained from American Type Culture Collection (Rockville, MD).

Isolation of CD34+ stem cells and culturing towards neutrophils

CD34+ cells were isolated as previously described. In brief, mononuclear cells were isolated from umbilical cord blood by density centrifugation over isotonic ficoll solution (Pharmacia, Uppsula, Sweden). Immunomagnetic selection with hapten conjugated antibody against CD34 was used to isolate CD34+ cells (Miltenyi Biotech, Auburn, USA). CD34+ cells were cultured in Iscove’s Modified Dulbecco’s Medium (Gibco, Paisley, UK) supplemented with 10% FCS, 50 µM β-mercaptoethanol, 10 U/ml penicillin, 10 µg/ml streptomycin, and 2mM glutamine at a density of 2-3x10^5 cells/ml. Cells were differentiated towards neutrophils upon addition of SCF (50 ng/ml), FLT-3 (50 ng/ml), GM-CSF (0.1 nmol/l), IL-3 (0.1 nmol/l), and G-CSF (30 ng/ml). Every 3-4 days, cells were counted and fresh medium was added to a density of 5x10^5 cells/ml. After 3 days of differentiation, cells were cultured further with IL-3 and G-CSF and from day 7 cells were cultured in the presence of G-CSF or with G-CSF and IL-3 as indicated. In some experiments, inhibitors were added upon isolation, and similar to the cytokines, refreshed every 3-4 days.

Morphological analysis of differentiating stem cells

Morphology of differentiating stem cells was analyzed by May-Grünwald Giemsa staining. During the maturation process cytospins were prepared from 10^5 cells. Slides were dried and fixed for 10 minutes in 100% methanol and May-Grünwald Giemsa staining was performed. In short, cytospins were stained in a 50% Eosin Methylene Blue solution for 20 minutes according to May-Grünwald (Sigma-Aldrich GmbH, Seelze, Germany). These were subsequently rinsed in water for 5 seconds, and the nuclei were counter-stained with 10% Giemsa solution (Merck, Darmstadt, Germany) for 15 minutes. Cells were imbedded in entellan (Merck, Darmstadt, Germany) and covered by a glass coverslip for preservation. Based on cytoplasmic staining and morphology of the nuclei, cells were counted as belonging to: 1) premature cells (which include blast cell and promyelocytes); 2) myelocytes (which include myelocytes and banded cells); 3) segmented cells, and 4) highly segmented cells.

Analysis of neutrophil differentiation markers

Lactoferrin positive cells were detected using a PE-conjugated antibody against lactoferrin (Beckman Coulter, Mijdrecht, The Netherlands). Staining was performed as described in the manufacturer's protocol, although adapted for stem cells. In brief, 3x10^5 cells were first washed in PBS and resuspended in 100 µl IntraPrep fixation reagent. After 15 minutes of incubation at room temperature, cells were washed and gently resuspended in 100 µl IntraPrep permeabilisation reagent. After 5 minutes of incubation, anti-lactoferrin-PE was added and incubated for another 15
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minutes. Cells were washed and resuspended in PBS containing 0.5% formaldehyde. Number of lactoferrin positive cells were detected by FACS analysis (FACSVantage, Becton Dickinson).

CD11b positive cells were analyzed using the mouse monoclonal antibody 44A against CD11b. 10^5 cells were collected and washed in cold PBS2+ (PBS, 10% GPO, 10% sodium citrate). Cells were incubated for 30 minutes on ice with anti-CD11b. Subsequently, cells were washed, and incubation was performed with a secondary goat anti mouse FITC labeled antibody (Becton Dickinson, San Jose, CA) for 30 minutes on ice. Cells were washed and CD11b positive cells were detected by FACS analysis (FACSVantage, Becton Dickinson).

Analysis of respiratory burst

The production of reactive oxygen species was measured by dihydrorhodamine staining. 400 µl of 1.25x10^6 cells/ml were as indicated first primed with GM-CSF (10^-10M) for 20 minutes at 37°C. Dihydrorhodamine 123 (DHR123) (Molecular Probes, Eugene, OR, USA) was added to a final concentration of 100 ng/ml and incubated for 10 minutes at 37°C. Respiratory burst was initiated by fMLP (10^-8M) or PMA (100ng/ml). Stimulation was stopped by washing with cold PBS2+. Cells were resuspended in PBS2+ and analyzed by FACS.

Measurement of apoptosis and proliferation.

Apoptosis of differentiating stem cells was measured by analyzing Annexin V-fluorescein isothiocyanate (FITC) binding (Bender Medsystems; Vienna, Austria). 10^5 cells were washed with PBS and resuspended in binding buffer (10 mM HEPES NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl2). Subsequently cells were incubated with Annexin V-FITC for 15 minutes at room temperature in the dark, washed with PBS and resuspended in binding buffer. Propidium iodide was added (1µg/ml) and the percentage of apoptotic cells were detected by FACS analysis (FACSVantage, Becton Dickinson). For proliferation assays, the number of viable cells were counted by trypan blue exclusion. Cell cycle analysis was determined as previously described 22. 3x10^5 cells were fixed in 70% ethanol at –20°C for 2 hours. Cells were gently spun down and permeabilized in 200µl 0.1% Triton X-100, 45mM Na2HPO4 and 25mM sodium citrate at 37°C for 20 minutes. Next, 750µl of 0.1% Triton X-100, 10mM PIPES, 2mM MgCl2, 40µg/ml RNase and 20µg/ml propidium iodide, was added and incubated for 30 minutes in the dark. DNA content was analyzed by FACS.

Results

G-CSF delays proliferation and enhances neutrophil differentiation

To investigate the role of different cytokine-activated signaling pathways in human neutrophil maturation, we developed a system in which myeloid progenitor cells can be differentiated to mature neutrophils ex-vivo. CD34+ hematopoietic stem cells (HSCs), enriched from human umbilical cord blood (UCB), were cultured as described in Material and Methods. Cells were passaged at day six and differentiated towards neutrophils in the presence of IL-3/G-CSF, or G-CSF alone and the effect on both proliferation and differentiation was analyzed. Culturing HSCs with IL-3/G-CSF resulted in a 565.2 ± 79.7 fold increase in cell number at day 16. However, treatment with G-CSF alone, resulted in greatly reduced proliferation of CD34+ stem cells (348.1 ± 29.0) (Fig. 1A).

To quantify the neutrophilic differentiation of stem cells treated with IL-3/G-CSF, or G-CSF alone, we analyzed the percentage of lactoferrin positive cells, a neutrophil specific granule protein, and CD11b expression on the cell surface of neutrophils. Treatment of G-CSF compared to the combined treatment of IL-3/G-CSF resulted in a dramatic increase in lactoferrin positive cells to 36.7%, compared with 18.6% at day 13 (Fig. 1B). The amount of lactoferrin positive cells in the G-CSF treated population further increased to 46.7% at day 16, whereas in the combined treatment the number of lactoferrin positive cells did not increase further. Analysis of CD11b expression demonstrated greatly enhanced levels at day 16 when cells were treated with G-CSF alone, compared to the population treated with IL-3/G-CSF (Fig. 1C).
Figure 1: Effect of cytokine treatment on the maturation of HSCs towards neutrophils. CD34+ cells isolated from cord blood were cultured for the first three days with a cocktail of cytokines as described in Materials and Methods. At day three only IL-3 and G-CSF were added, at day six cells were divided and cultured further with IL-3 and G-CSF or with G-CSF alone. (A) Proliferation was analyzed by counting number of viable cells every 3-4 days and fold proliferation was calculated. (B) Differentiation was analyzed by detection of lactoferrin positive cells at day 9, 13, and 16 as described in Materials and Methods. The percentage of highly lactoferrin positive cells, gated as shown, was determined by FACS analysis at 9, 13 and 16 days as described in Materials and Methods. (C) Expression of CD11b on the cell surface of differentiating cells was detected by FACS analysis. The percentage of CD11b positive cells, gated as shown in the first figure, are indicated. (D) Determination of differentiation stage was analyzed by May-Grunwald Giemsa staining and cells were counted as described in Materials and Methods. Representative pictures of differentiated cells at day 16 are also depicted. Graphs represent mean percentage cells of different stages. All data represent the mean of four independent experiments.

Histological analysis of neutrophil differentiation was determined by May-Grunwald Giemsa staining as described in Materials and Methods. Stem cells treated with G-CSF alone exhibited a more mature neutrophil phenotype, including segmented and highly segmented cells, which represented almost 53.7% of the total population (Fig. 1D). In contrast, cells treated with IL-3/G-CSF contained only 26.7% of mature neutrophilic cells (Fig. 1D). In Figure 1D representative pictures of cells after 16 days of differentiation are depicted for both conditions. These results demonstrate that the combined IL-3 and G-CSF treatment drives the expansion of the HCS more efficiently than G-CSF, whereas G-CSF treatment gives rise to more terminally differentiated neutrophils.

Production of reactive oxygen species correlates with enhanced differentiation
Although we have demonstrated that treatment with G-CSF alone enhances neutrophil differentiation of HSC, as determined by the expression of granulocytic markers, and histological staining, this doesn’t measure functional maturity of these cells. Thus, we further analyzed the ability of differentiated neutrophils to produce reactive oxygen species (ROS). ROS production by the so-called “respiratory burst” is a complex process that requires correct
assembly of the NADPH oxidase enzyme complex. The NADPH oxidase is a multi-subunit complex composed of five components: flavocytochrome \(b_{558}\), p47\(_{phox}\) and p67\(_{phox}\) and p40\(_{phox}\) and the small GTPase Rac2, which all have to be present for ROS production. We first tested Phorbol Myristate Acetate (PMA) induced ROS production, as an indication of the functionality of the NADPH oxidase complex. After six days of differentiation, no ROS production was observed in response to addition of PMA (Fig. 2A). However by day nine, the required components of the NADPH oxidase complex were apparently present since PMA-treatment resulted in ROS production (Fig. 2A). Comparison of cells differentiated with the different cytokine combinations demonstrated greatly enhanced ROS production with G-CSF treatment alone compared to combined IL-3/G-CSF treatment, suggesting terminal neutrophilic differentiation. A more physiological method of inducing the respiratory burst was tested utilizing the chemoattractant fMLP, a potent activator of ROS formation in pre-activated or “primed” neutrophils. Differentiated cells were first primed with GM-CSF for 20 minutes prior to stimulation with fMLP. HSCs differentiated towards neutrophils in the presence of G-CSF alone showed a greatly increased response to fMLP treatment compared to treatment with both IL-3/G-CSF (Fig. 2B). In conclusion, G-CSF regulates the differentiation of HCS towards functionally mature neutrophils more efficiently when IL-3 induced signaling pathways are not concomitantly activated.

Figure 2: Analysis of the production of reactive oxygen species.

The ability of differentiated cells to produce reactive oxygen species was analyzed by DHR123 staining as described in Materials and Methods. Cells were differentiated for 16 days with IL-3/G-CSF or G-CSF. (A) PMA induced formation of reactive oxygen species during differentiation. Unstimulated cells of the same day were used as a control. (B) Respiratory burst induced by fMLP at day 16. Cells were pre-activated with GM-CSF for 20 minutes prior to addition of fMLP. Values below graphs indicate the median difference of PMA or fMLP treated cells versus untreated cells of the same population. Data are representative of three independent experiments.

Inhibition of PI3K induces cell cycle arrest and blocks differentiation to neutrophils.

PI3K has been linked to regulation of proliferation and survival in many cell systems. However, little is known concerning the role of PI3K in the differentiation of human hematopoietic cells. We investigated the role of PI3K on G-CSF induced maturation of CD34\(^+\) stem cells utilizing the specific pharmacological inhibitor LY294002. Differentiation towards the myeloid lineage was initiated by a cytokine cocktail as described in Materials and Methods. Treatment with LY294002, totally abrogated proliferation (Fig. 3A) and this was not due to enhanced cell death since we observed only a very modest elevation in the amount of apoptotic cells as analyzed by annexin-V binding (Fig. 3B). Analysis of the DNA content of cells demonstrated a decrease in the number of dividing cells in the presence of LY294002 within 24 hours of treatment (Fig. 3C). Additionally we analyzed whether the arrested cells were still able to undergo differentiation induced by G-CSF. Cells were cultured for 13 days and lactoferrin expression and histological staining were analyzed. No lactoferrin positive cells were observed in the LY294002 treated cells (Fig. 3C). Histological staining demonstrated a lack of mature neutrophils (Fig. 3D). Taken together these results indicate that PI3K activity is critical for regulating proliferation as opposed to survival of CD34\(^+\) stem cells, as well as playing an important role in the maturation towards neutrophils.
Figure 3: The effect of LY294002 on the proliferation and differentiation of hematopoietic progenitor cells.

CD34+ stem cells were cultured in the absence or presence of 20 µM LY294002. (A) Proliferation of CD34+ stem cells was analyzed by counting viable cells after 24, 48 and 72 hours. (B) The percentage of apoptotic progenitor cells was determined after 24, 48 and 72 hours. Percentage of early apoptotic cells (annexin-V positive, PI negative) and percentage of late apoptotic cells (annexin-V and PI positive) are indicated. Numbers of viable cells are indicated as annexin-negative cells (right panel). (C) Cell cycle profiles are depicted of cells cultured for 24 and 48 hours with or without LY294002. (D) The effect of LY294002 on the differentiation of HCS towards neutrophils. Hematopoietic progenitor cells were cultured for 15 days without (control) or with LY294002. Representative dotplots of lactoferrin staining, as gated in Figure 1B, are indicated. Graphs represent the mean percentage of lactoferrin positive cells of three independent experiments (E) Determination of differentiation stage was analyzed by May-Grunwald Giemsa staining as described in Figure 1D. Graphs represent the mean percentage cells of the different stages. All data are representing the mean of three independent experiments.
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Figure 4: Effect of PD89059 on the maturation of HCS towards neutrophils. CD34+ stem cells were differentiated with G-CSF and cultured in the absence or presence of 20µM PD89059. (A) Proliferation of CD34+ stem cells was analyzed by counting viable cell numbers (B) Differentiation was analyzed by lactoferrin staining. Representative dotplots showing the percentage of lactoferrin positive cells, as gated in Figure 1B, are shown. Graphs represent the mean percentage of lactoferrin positive cells of three independent experiments (E) Determination of differentiation stage was analyzed by May-Grunwald Giemsa staining as described in Figure 1D. Graphs represent the percentage cells of the different stages. All data represent at least three independent experiments.

Inhibition of MEK reduces proliferation without effecting neutrophil differentiation

Another well-characterized signaling pathway downstream of multiple cytokine receptors is the Ras-Raf-MEK-MAPK pathway. It has been suggested that this pathway is involved in the G-CSF induced regulation of the cell cycle in myeloid cell lines. However, little is known concerning the role of this pathway in the differentiation of human hematopoietic progenitors towards neutrophils. For this purpose, we differentiated CD34+ stem cells with G-CSF in the presence or absence of the specific MEK inhibitor PD98059. We observed a growth inhibitory effect of PD89059 on treated cells, with a reduction to about 30% of the number of cells compared to the control at day 16 (Fig. 4A). Analysis of the role of MEK inhibition on differentiation demonstrated no significant differences in the percentage of lactoferrin positive cells (Fig. 4B) or the number of CD11b positive cells (data not shown). Morphologic analysis by May-Grunwald Giemsa staining in the PD89059 treated cells compared to the control cells also demonstrated no reduction in the number of mature, differentiated cells (Fig. 4C). Taken together these data suggest a role for MEK in regulating proliferation, but no significant role in the differentiation of hematopoietic progenitors towards neutrophils.
Discussion

The production of mature blood cells, hematopoiesis, involves a carefully orchestrated series of events involving self-renewal and differentiation of primitive pluripotent stem cells. There is a growing understanding of the complexity of the intrinsic and extrinsic controls built into this system to maintain its integrity. The external influences are many and diverse, encompassing interactions of developing hematopoietic cells with extracellular matrix molecules, a variety of stromal cell types and with growth factors. A large family of hematopoietic cytokines is able to control the proliferation, differentiation and survival of progenitor cells. Interleukin (IL)-3, for example, stimulates the growth of multipotent progenitor cells that will eventually differentiate into granulocytes, macrophages, megakaryocytes and erythrocytes. Their ability to stimulate proliferation works primarily during early hematopoiesis without specifying the final differentiation fate of the progenitor. Other cytokines act at a later stage to direct the final fate of a cell. G-CSF is, for example, critical for the terminal differentiation of myeloid progenitors to neutrophils, and granulocyte-macrophage colony-stimulating factor (GM-CSF) has its actions restricted primarily to neutrophil and monocyte lineages. These late acting cytokines are thought to initially stimulate cellular proliferation followed by differentiation to a non-proliferating mature cell. It is the combinations of cytokines acting on a particular hematopoietic progenitor that will initiate a specific developmental program determining the final differentiation fate of the target cell. These signals result in the activation of distinct downstream signal transduction pathways resulting in changes in the regulation of expression, or activation of several families of transcription factors.

In this study, we developed an ex-vivo model system in which cytokine induced differentiation of human hematopoietic progenitors towards neutrophils could be investigated. Maturation in the presence of IL-3 together with G-CSF resulted in increased cell expansion, however under these conditions the number of mature neutrophils was considerably lower, compared to cells treated with G-CSF alone (Fig. 1). This suggests that these two cytokines induce distinct signaling pathways regulating proliferation and/or differentiation. However, data from various cell lines have shown that the intracellular pathways activated by the G-CSF receptor and the IL-3 receptor are highly overlapping \(^8; 47-50\). The differences observed between these two cytokines could be explained due to differences in signal strength, or possibly the duration of activation of various intracellular signaling pathways. These differences have been observed in the rat pheochromocytoma PC12 cell line stimulated with either epidermal growth factor (EGF) or nerve growth factor (NGF) (reviewed in \(^51\) and \(^52\)). Both factors induce similar intracellular signal transduction pathways, however EGF induces proliferation, while NGF induces differentiation. However, in this system NGF induces a sustained, whereas EGF induces transient ERK activation. This has led to the proposal that the duration of ERK activation is important for determining whether a particular stimulus may result in a differentiation or proliferation response. Since both IL-3 and G-CSF induce PI3K activation but only G-CSF triggers neutrophil differentiation, a similar mechanism might be possible.

The results obtained from the comparison of G-CSF treatment versus a combined IL-3/G-CSF treatment suggests that decreased proliferation is linked to increased differentiation and vice versa. Indeed it has been reported that over-expression of CDK inhibitors p27\(^{kip1}\) and p21\(^{Cip1}\) in the absence of differentiation agents can lead to terminal differentiation of promonocytic cells (U937) \(^53\). Furthermore, myeloid progenitor cells isolated from p27\(^{kip1}\)-deficient mice show significantly increased proliferation and reduced differentiation in response to G-CSF \(^54\). Additionally, it has been described that retinoic acid induced differentiation of human myeloid cell lines correlates with cell cycle arrest, associated with sequential down regulation of cyclin E, c-myc and upregulation of p27Kip1 \(^55\). However our data, utilizing the PI3K inhibitor LY294002, demonstrate that a cell cycle arrest per se is not in itself sufficient to induce differentiation (Fig. 3). In the maturation of megakaryocytes, thrombopoietin or e-kit ligand can promote proliferation and differentiation at the same time \(^56; 57\). An additional study demonstrated that Insulin Growth factor 1 (IGF-I) increased the early development of vitamin D3 induced macrophage differentiation, which was associated with elevated cyclin E levels, hyperphosphorylated Rb protein, and suppression of p27\(^{Kip1}\) levels \(^58\). These data,
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Together with our findings suggest that the initial steps of differentiation can occur concomitantly with the progression through the cell cycle.

Although a role for PI3K in cellular proliferation has been well established (reviewed in 40), little is known concerning the potential role of PI3K in differentiation. Mice lacking the regulatory p85α subunit of PI3K have reduced numbers of mature B-cells 59-60. One mechanism by which PI3K might regulate differentiation is through regulation of the Retinoblastoma protein (Rb) during the maturation process. Rb mediates a block in the G1-phase of the cell cycle by inhibiting the E2F family of transcription factors, thereby preventing transcription of genes necessary for further progression in the cell cycle 61. Phosphorylation of Rb results in release of Rb from E2F and transcription can proceed. While having a clearly defined role in transcriptional regulation of the cell cycle, Rb proteins have also been shown to direct the differentiation program in myoblasts, adipocytes and hematopoietic cells, since these cells fail to differentiate in the absence of Rb 62. Furthermore, high levels of Rb are induced and sustained during erythroid differentiation while Rb is down regulated during granulocytic maturation 63. PI3K has been previously implicated in regulating E2F-mediated transcription through Rb 64-66. Additionally, a link between PI3K and p130 Rb-like protein levels has been described 67. In this manner PI3K might be able to regulated differentiation through regulation of Rb phosphorylation and protein levels. Figure 5, schematically represents the role for PI3K in both proliferation and differentiation during the maturation process. While multiple cytokines and growth factors, such as IL-3, are unable to induce differentiation, they are potent in activating PI3K. Therefore PI3K activity alone is unlikely to be sufficient to induce differentiation.

We have also demonstrated a role for MEK in regulating the proliferation of human CD34+ progenitors towards neutrophils. A potential mechanism underlying this observation for MEK is through the upregulation of cyclin D1 expression 30. Sustained activation of ERK1 by MEK was demonstrated to be required for the continued expression of cyclin D1 in the G1 phase of the cell cycle. Another possible target for MEK is the transcription factor c-myc, which is a critical regulator of cellular proliferation 68. Indeed, it has previously been demonstrated that MEK is required for CSF-1 mediated c-myc transcription and cytokine induced proliferation 69. Activation of ERK by MEK also results in the direct phosphorylation of c-myc, which acts to stabilize the protein 70. c-myc stability can also be increased through PI3K by inhibiting GSK-3, a kinase which itself can phosphorylates c-myc, targeting it for ubiquitination 70. A recent study has analyzed the effect of mutation of tyrosine 764 in the intracellular region of the G-CSF in primary mouse bone marrow cells. Cells expressing this G-CSFR mutant responded with reduced proliferation in response to G-CSF, without having an effect upon differentiation 71. This mutation was also associated with a reduced ERK activity, further supporting a critical role for the MEK-ERK pathway in proliferation (Fig. 5).

In this study, we have analyzed the role of various cytokine-induced intracellular signaling pathways in an ex-vivo differentiation model utilizing primary human hematopoietic stem cells. We have demonstrated critical roles for PI3K and MEK in neutrophil maturation, a process involving control of both proliferation and differentiation. Importantly, we have identified PI3K as a novel player in the process of myeloid differentiation. Our data also suggest that cell cycle arrest per se does not result in induction of an intrinsic neutrophil differentiation program. Further work will be required to understand the molecular mechanisms underlying these observations.

Figure 5. Schematic representation of the role of PI3K and MEK in maturation of hematopoietic stem cells. PI3K has a critical role in both proliferation and differentiation. Although PI3K is necessary in these processes, it is probably not sufficient to induce differentiation. MEK has a significant role only in proliferation.
References


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