Role of Erk1/2 Signaling in the Regulation of Neutrophil Versus Monocyte Development in Response to G-CSF and M-CSF*

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Background: G-CSF and M-CSF are cytokines that support the development of neutrophils and monocytes, respectively. The duration of Erk1/2 activation by G-CSF and M-CSF affects the lineage commitment of myeloid precursors. G-CSF and M-CSF instruct neutrophil versus monocyte development through differential activation of Erk1/2.

Results: The expression and activities of lineage specific transcription factors. Monocyte and neutrophil lineage specifications require the transcription factors C/EBPα and PU.1 that are components of a myeloid transcriptional regulatory circuit, which includes Egr1, Egr2, Nab2, and Gfi1, among others (5, 6). A high C/EBPα/PU.1 ratio supports neutrophil development whereas increased expression of PU.1 favors monocyte over granulocyte lineage decision (7). G-CSF and M-CSF instruct neutrophil cell fate in part through activating Gfi1 that promotes neutrophil development and suppresses the alternative monocyte development (8–10). PU.1 acts in a graded manner to direct distinct cell fates with a high expression promoting monocyte development and a low expression required for B lymphocyte development (11). PU.1 activates IRF8, Klf4, Egr2, and Nab2 that direct monocyte development at the expense of neutrophil cell fate (12–14). In addition, transcription factors c-Fos and c-Jun have been shown to positively regulate monocyte development (5, 15, 16).

Conclusions: G-CSF and M-CSF are two lineage-specific hematopoietic cytokines that play a dominant role in granulopoiesis and monopoiesis, respectively. Hematopoietic cytokines have been shown to positively regulate monocyte development (5, 15, 16).

Significance: This reveals a key mechanism by which G-CSF and M-CSF control lineage specification.
G-CSF in Neutrophil Lineage Specification

into distinct types of mature blood cells. While both models are backed by experimental data, two recent reports lend strong support to the instructive model, at least for G-CSF and M-CSF. Using the bio-imaging approaches that permit continuous long-term observation at the single-cell level, it was shown that G-CSF and M-CSF can instruct myeloid lineage choice in HSCs and GMPs (20, 21). However, the intracellular signaling mechanisms by which G-CSF and M-CSF instruct granulocyte versus monocyte lineage commitment are unknown.

In this report, we show that substitution of Tyr-729 of G-CSF with phenylalanine (F) resulted in monocyte development in response to G-CSF, which was associated with prolonged activation of Erk1/2 and augmented activation of c-Fos and Egr1. Treatment of cells with Mek1/2 inhibitors or knockdown of c-Fos or Egr1 essentially rescued neutrophil development. Notably, the Mek1/2 inhibitors also promoted neutrophil development at the expense of monocyte formation induced by M-CSF. Our data reveal an important signaling mechanism by which G-CSF and M-CSF direct neutrophil versus monocyte lineage specification.

Experimental Procedures

Cell Lines and Cell Culture—Murine myeloid 32D cells expressing the different forms of G-CSF have been described (22, 23). Cells were maintained in RPMI 1640 with 10% heat-inactivated fetal bovine serum (HI-FBS), 10% WEHI-3B cell-conditioned media as a crude source of murine interleukin-3, and 1% penicillin/streptomycin (P/S). Murine multipotential FDCP-mix A4 cells (24) were maintained in IMDM medium supplemented with 15% horse serum and 10% WEHI-3B cell-conditioned medium. FDCP-mix A4 cells were transfected with the human G-CSFR expression constructs by electroporation and then selected in G418 (0.6 mg/ml). Cells expressing the human G-CSFR were isolated by fluorescence-activated cell sorting (FACS) following staining with an anti-human G-CSFR antibody (BD Biosciences, San Jose, CA).

Flow Cytometry—Cells were washed in PBS with 2% horse serum and blocked with Fc block (eBioscience) for 15 min. Cells were then incubated with isotype control anti-mouse IgG antibody conjugated with phycoerythrin (PE), anti-mouse IgG antibody conjugated with fluorescein isothiocyanate (FITC) or PE-conjugated anti-F4/80 antibody for 30 min prior to washing in PBS with 2% horse serum. All antibodies were purchased from eBioscience. Samples were analyzed by flow cytometry using a FACSCalibur and the CellQuest software system (BD Biosciences).

Western Blot Analysis—Cells were lysed in SDS lysis buffer and proteins were separated by SDS-PAGE prior to transfer onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with the appropriate antibodies and signals were detected by enhanced chemiluminescence. The antibodies against phospho-Stat5, phospho-Stat3, phospho-Erk1/2, c-Fos, phospho-c-Fos (Ser32), Egr-1, and β-actin were purchased from Cell Signaling.

Luciferase Reporter Assay—Cells were transfected with the reporter constructs TRE5-tk-Luc (a gift from Dr. Lirim Shemshedini, The University of Toledo) or pEBS2-Luc (a gift from Dr. Gerald Thiel, University of the Saarland Medical Center). Sixteen hours after transfection, cells were washed and stimulated with G-CSF (10 ng/ml) for 8 h. Luciferase activities were measured using the Molecular Devices Lmaxluminometer (Sunnyvale, CA).

RNA Interference—Lentiviral constructs containing murine c-Fos and Egr-1 shRNAs were purchased from Thermo Scientific. Lentiviral vector encoding a murine ERK2 shRNA (TRCN54729) was purchased from Dharmacon. To target murine ERK1, oligonucleotides were designed to generate a mature antisense AATGTAACATCTCTCATGTC and cloned into pLKO.1-Hygro (Addgene plasmid 24150). 293T cells were transfected with the lentiviral constructs along with packaging plasmids psPAX2 and pMD2G using the calcium phosphate coprecipitation procedure. Supernatants containing viral particles were harvested at 48 and 72 h post-transfection, concentrated, and used to infect cells in the presence of 8 µg/ml polybrene (Santa Cruz Biotechnology). Cells were selected in 2 µg/ml puromycin for 48 h or hygromycin (1 mg/ml) for 4 days prior to evaluation of gene knockdown by Western blot analysis.

Real-time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)—Total RNA was extracted using TRIzol reagent (Invitrogen), and cDNA was synthesized using the GoScript™ Reverse Transcription System and Oligo(dT)15 primer (Promega, Madison, WI). qRT-PCR was performed using the SsoFast™ EvaGreen Supermix® kit (Bio-Rad), and the relative levels of mRNAs for the different myeloid differentiation markers were normalized to GAPDH mRNA expression.

Bone Marrow (BM) Cell Culture—BM cells were isolated from the long bones of 6–8-week-old C57BL/6 mice and red blood cells were lysed with ACK Lysing buffer (Lonza). Cells were then subjected to lineage depletion using the antibodies against the following lineage markers: CD3e, CD11b, CD45R/B220, Ly6G, and Ly-6C, and TER-119 (BD Biosciences) and immunomagnetic beads (Miltenyi Biotech). Lineage negative (Lin−) cells were cultured in IMDM media with 10% HI-FBS, 10 ng/ml IL-3, 20 ng/ml IL-6, and 25 ng/ml SCF (Peprotech). For M-CSF-induced differentiation, cells were cultured in IMDM medium with 10% HI-FBS, 10 ng/ml IL-3, 20 ng/ml IL-6, 25 ng/ml SCF, and 10 ng/ml M-CSF for 3 days prior to evaluation of cell differentiation. For colony forming assays, 6–8-week-old C57BL/6 mice were treated with 5-fluorouracil (5-FU; 150 mg/kg). BM cells were isolated 5 days later and incubated in IMDM media with 10% HI-FBS, 10 ng/ml IL-3, 20 ng/ml IL-6, 25 ng/ml SCF for 1 h for recovery. Cells (10⁴) were then seeded in methylcellulose-based medium (R&D System) with 10% HI-FBS, IL-3, IL-6, SCF, and M-CSF with or without indicated inhibitors. Colonies were enumerated on day 7.

Apoptosis Assay—Apoptosis was examined using the Annexin V-PE apoptosis detection kit (BD Biosciences). Briefly, 0.3 × 10⁶ cells were collected and incubated with Annexin V-PE and 7 amino-actinomycin (7-AAD). Cells were analyzed by two-color flow cytometry.

Statistics—GraphPad Prism software (GraphPad Software, La Jolla, CA) was used for all statistical analysis. Data are shown as mean ± S.D. in all figures. A p value of <0.05 was considered significant for all analyses and shown as *. ** denotes p < 0.01, *** denotes p < 0.001, and **** denotes p < 0.0001.
Results

Tyrosine 729 of G-CSFR Is Essential for Instructing Neutrophil Lineage Choice—The human G-CSFR contains four tyrosine (Y) residues in the cytoplasmic domain, i.e., Y704, Y729, Y744, and Y764 (Fig. 1A), that have been implicated in G-CSF-stimulated proliferation, survival and differentiation (23, 25–28). Notably, Y729, Y744, and Y764 are located in the C-terminal region of G-CSFR required for differentiation signaling (22, 29). To address the roles of the C-terminal tyrosine residues in G-CSF response, we evaluated the G-CSF response of 32D cells transfected with the G-CSFR mA mutant (32D/mA) in which the three C-terminal tyrosine (Y) residues were mutated to phenylalanine (F) (23, 30). As reported previously (22, 31), 32D cells expressing the wild
FIGURE 3. Tyrosine 729 of G-CSFR is required for neutrophil development in FDCP-mix A4 cells. Cells were transfected with the WT or Y729F form of G-CSFR and evaluated for G-CSFR expression by flow cytometry (A). Cells were then cultured in G-CSF for 2 days prior to evaluation of growth behaviors (B), morphological features (C), surface expression of F4/80 (D), and the levels of M-CSF and Mmp-12 mRNAs (E) except that the morphology of FDCP/GR cells was examined on day 5.

FIGURE 4. G-CSFR Y729F mediates prolonged activation of downstream signaling pathways. 32D (A and C) and FDCP-mix A4 (B and D) cells expressing WT or Y729F form of G-CSFR were starved for 2 h prior to stimulation with G-CSF for indicated minutes (A and B) or hours (C and D). Activation of Stat5, Akt, and Erk1/2 was examined by Western blot analysis using phospho-specific antibodies.
type (WT) G-CSFR (32D/GR) underwent terminal neutrophilic differentiation after culture in G-CSF for 6 to 9 days. Interestingly, upon culture in G-CSF, 32D/mA cells showed increased cell sizes, adherence to the culture dishes and morphological features characteristic of monocytes (Fig. 1, B and C). Consistent with this, the surface expression of macrophage marker F4/80 and the mRNA levels of M-CSF and Mmp-12 were significantly higher in 32D/mA cells than in 32D/GR cells (Fig. 1, D and E). Thus, mutations of the C-terminal tyrosine residues of G-CSFR resulted in monocyte rather than neutrophil development in response to G-CSF, suggesting that the C-terminal tyrosine residues function to promote neutrophil cell fate and suppress the alternative monocyte development.

To identify the involved tyrosine residue, we evaluated the G-CSF responses of 32D cells transfected with the G-CSFR mutants containing individual Y-to-F substitutions in the C terminus (Fig. 1A). Y744F and Y764F mutations had no significant effect on neutrophil development; however, 32D cells expressing G-CSFR Y729F (32D/Y729F) displayed features associated with monocyte development (Fig. 2). To confirm the observations in a different cell line, we expressed the WT G-CSFR and Y729F mutant in murine multipotent FDCP-mix A4 cells. Parental FDCP-mix A4 cells expressed no detectable levels of endogenous G-CSFR and M-CSFR on cell surface, and the cells died in G-CSF or M-CSF within 24 h (data not shown). FDCP-mix A4 cells transfected with the WT G-CSFR (FDCP/GR) developed into mature neutrophils after culture in G-CSF for 6 days (Fig. 3). In contrast, FDCP/Y729F rapidly developed into monocytes within 2–3 days following G-CSF induction and died subsequently. Thus, G-CSFR Y729 is required for neutrophil development in response to G-CSF.

Prolonged Activation of Erk1/2 Is Required for Monocyte Development Mediated by G-CSFR Y729F—We previously showed that Y729 of G-CSFR controls the duration of G-CSFR signaling (23). The kinetics of G-CSF-stimulated activation of different downstream pathways was examined in more detail in 32D and FDCP-mix A4 cells. As shown in Fig. 4, A and B,
G-CSFR Y729F mediated prolonged activation of Stat5, Akt, and Erk1/2 in both cell lines. Activation of these pathways was also enhanced in FDCP-mix A4 cells. It has been shown that M-CSF stimulates more potent activation of Erk1/2 compared with G-CSF and that sustained activation of Erk1/2 is required for M-CSF-induced monocytic differentiation (32, 33). We therefore examined the activation status of Erk1/2 in cells continuously cultured in G-CSF for up to 48 h. Erk1/2 phosphorylation was barely detectable at 24 h in cells expressing the WT G-CSFR, but readily detected in cells expressing G-CSFR Y729F, and even at 48 h in 32D/Y729F cells (Fig. 4, C and D). Thus, prolonged activation of Erk1/2 led to their persistent activation when cells were cultured continuously in G-CSF.

We addressed whether inhibition of Erk1/2 activation rescued neutrophil development. As shown in Fig. 5, treatment of 32D/Y729F and FDCP/Y729F cells with the Mek1/2 inhibitor U0126 or PD0325901 resulted in the typical neutrophil development with reduced cell sizes, loss of adherent phenotype and diminished expression of M-CSF and Mmp-12 although the expression of F4/80 was not significantly altered. Comparable results were obtained with another Mek1/2 inhibitor PD98059 (data not shown). To further address the role of Erk1/2 pathway in monocyte development mediated by G-CSFR Y729F, we knocked down their expression using shRNAs specifically targeting murine Erk1 and Erk2 (32). As shown in Fig. 6, knockdown of Erk1 and Erk2 largely rescued neutrophil development in response to G-CSF in both 32D/Y729F and FDCP/Y729F cells. Thus, prolonged activation of Erk1/2 pathway is essential for monocytic development directed by G-CSFR Y729F.

**Suppression of Erk1/2 Pathway Favors Neutrophil Development at the Expense of Monocyte Cell Fate in Response to M-CSF**—Previous studies have provided supportive evidence for an instructive role of M-CSF in monocyte development (20, 21). However, how M-CSF instructs monocyte cell fate remains unresolved. We examined Erk1/2 activation by G-CSF and M-CSF in mouse Lin^- BM cells. Treatment with M-CSF, but not G-CSF, resulted in strong and sustained activation of Erk1/2 for at least six hours (Fig. 7A). In contrast, G-CSF, but not M-CSF, stimulated Stat3 phosphorylation. When cultured in M-CSF for 3 days, more than 70% of Lin^- BM cells developed into monocytes/macrophages (Fig. 7, B and C). Interestingly, addition of U0126 or PD0325901 to the cultures increased the numbers of mature neutrophils, but markedly suppressed macrophage development. The decrease in macrophage population was unlikely due to increased apoptosis as U0126 and PD0325901 had only a modest effect on cell survival (data not shown). We further performed colony formation assays to assess the effect of the Mek1/2 inhibitors on the development of myeloid precursors. As shown in Fig. 7D, U0126 and PD0325901 caused an approximate 2-fold increase in the number of CFU-G, but significantly decreased the number of CFU-M. In support of increased neutrophil development at the expense of macrophage development, the two Mek1/2 inhibitors downregulated the expression of M-CSF and Mmp-12, but upregulated the mRNA levels of the neutrophil differentiation markers, including the primary granule proteins myeloperoxidase (MPO) and neutrophil elastase (NE), secondary granule protein lactoferrin (LF) and tertiary granule protein gelatinase B in BM cells cultured in M-CSF (Fig. 7E). Thus, upon suppres-
sion of Erk1/2 signaling, M-CSF mainly supported neutrophil development in mouse BM cells.

**G-CSFR Y729F Mediates Enhanced Activation of c-Fos and Egr1**—c-Fos and Egr1 are immediate early genes (IEGs) that are activated by the Erk1/2 pathway (34, 35). When persistently activated, Erk1/2 also directly phosphorylates c-Fos that is rapidly induced following Erk1/2 activation and thus c-Fos phosphorylation serves as a sensor for Erk1/2 signal duration (36, 37). Erk1/2-mediated phosphorylation stabilizes c-Fos and primes additional phosphorylation by exposing an Erk1/2 docking site, called DEF domain. Interestingly, Egr1 also has a DEF domain and several potential Erk1/2 phosphorylation sites (37). We investigated whether prolonged activation of Erk1/2 mediated by G-CSFR Y729F led to enhanced phosphorylation and activation of c-Fos and Egr1. In 32D and FDCP-mix A4 cells expressing the WT or Y729F form of G-CSFR, G-CSF stimulation resulted in rapid induction of c-Fos and Egr1, accompanied by their electrophoretic mobility shifts that became increasingly more significant up to 8 h after G-CSF stimulation (Fig. 8A). Both U0126 and PD0325901 blocked c-Fos and Egr1 induction by G-CSF (data not shown). Notably, the induction of c-Fos and Egr1 was more sustained, and their mobility shifts greater in cells expressing G-CSFR Y729F than in cells expressing WT G-CSFR. Consistent with this, phosphorylation of c-Fos on serine 32, which occurred one hour after c-Fos induction, was greater and more sustained in cells expressing G-CSFR Y729F.

Egr1 has been shown to promote monocyte development at the expense of neutrophil cell fate (14, 16, 38). A potential role of c-Fos in monocyte development has also been proposed (5, 15, 39). We next examined whether sustained induction and phosphorylation of c-Fos and Egr1 enhanced their transcri-
c-Fos heterodimerizes with Jun family transcription factors to form AP-1 proteins that activate transcription of target genes via the tetradecanoyl phorbol acetate response element (TRE). In both 32D and FDCP-mix A4 cells, G-CSFR Y729F mediated augmented activation of the luciferase reporter construct containing three repeats of TRE (40).

**FIGURE 8.** G-CSFR Y729F mediates augmented activation of c-Fos and Egr1. A, 32D (left panels) and FDCP-mix A4 (right panels) cells expressing WT or Y729F form of G-CSFR were treated with G-CSF for different times and examined for c-Fos and Egr1 expression, and c-Fos phosphorylation on serine 32 by Western blot analysis. B and C, cells as indicated were transfected with the luciferase reporter constructs containing three repeats of TRE (B) or four repeats of Egr1 binding site (C). Eighteen hours later, cells were treated with IL-3 or G-CSF for 8 h prior to evaluation of luciferase activity. Data are presented as luciferase activity induced by G-CSF relative to that induced by IL-3.

**FIGURE 9.** Knockdown of c-Fos restores G-CSF-induced neutrophil development in 32D/Y729F cells. A, cells were transduced with empty lentiviral vector (Ctr) or two different c-Fos shRNAs (79 and 80), and examined for c-Fos expression. Cells were subsequently cultured in G-CSF for 6 days prior to evaluation of growth behaviors (B), morphology (C), M-CSF and Mmp-12 mRNA levels (D), and F4/80 expression (E).
Activation of the reporter construct with four repeats of conserved Egr1 binding site (41) was also augmented in cells expressing G-CSFR Y729F (Fig. 8C). As expected, activation of the two reporter constructs by G-CSF was inhibited by U0126 and PD0325901 (data not shown). Thus, prolonged activation of Erk1/2 was associated with augmented activation of AP1 and Egr1.

Neutrophil Development Is Partially Restored upon Knockdown of c-Fos or Egr1 in Cells Expressing G-CSFR Y729F—To address whether enhanced activation of c-Fos contributed to monocyte development, we transduced 32D/Y729F cells with the lentiviral constructs containing two different shRNAs against c-Fos. shRNA 79 markedly and shRNA 80 moderately inhibited c-Fos induction by G-CSF (Fig. 9A). When cultured in G-CSF for 6 days, the c-Fos knocked down 32D/Y729F cells developed into morphologically mature neutrophils with significantly less adherence to culture dishes and reduced expression of M-CSF and Mmp-12 (Fig. 9, B–D). Notably, as in cells treated with U0126 and PD0325901, the expression of F4/80 was not altered by c-Fos knockdown (Fig. 9E). To address the role of enhanced Egr1 activation in monocyte development, we expressed 3 different Egr1 shRNAs in 32D/Y729F cells. As shown in Fig. 10A, shRNAs 25 and 26 inhibited Egr1 induction by G-CSF whereas shRNA 24 showed no effect. Notably,
shRNAs 25 and 26, but not shRNA 24 supported neutrophil development at the expense of monocyte development (Fig. 10, B–D). Similar to c-Fos knockdown, F4/80 expression was not affected following Egr1 knockdown (Fig. 10E).

We also knocked down c-Fos and Egr1 in FDCP/Y729F cells. As shown in Fig. 11, knockdown of either c-Fos or Egr1 resulted in a shift in cell morphology toward neutrophils following culture in G-CSF for 2 days, which was associated with down-regulation of M-CSF and Mmp-12, and up-regulation of NE and MPO. No significant changes in F4/80 expression were observed (data not shown). Thus, knockdown of c-Fos or Egr1 partially rescued neutrophil development in both 32D/Y729F and FDCP/Y729F cells. The data also indicated that c-Fos and Egr1 were not involved in the regulation of F4/80 expression.

Discussion

It has long been debated whether hematopoietic cytokines direct lineage specification and, if they do, little is known about the underlying signaling mechanisms. Two recent studies provide strong evidence for an instructive role of G-CSF and M-CSF in the regulation of lineage commitment toward neutrophils and monocytes, respectively, in GMPs and HSCs (20, 21). However, how G-CSF and M-CSF regulate the intracellular signaling pathways to resolve neutrophil versus monocyte cell fate decision is still unknown.

In this report, we have shown that G-CSFR Y729F promotes monocyte rather than neutrophil development. Interestingly, G-CSFR Y729F has previously been shown to induce macrophage-like morphology in murine myeloid L-GM cells (25) and support significantly increased formation of macrophage colonies but reduced number of granulocyte colonies in mouse primary BM cells (27). Our data further indicate that monocyte development directed by G-CSFR Y729F is associated with prolonged activation of Erk1/2 and inhibition of Erk1/2 signaling largely rescues neutrophil development. Importantly, M-CSF, but not G-CSF, induces sustained and strong activation of Erk1/2 pathway in Lin−/H11002 BM cells, and inhibition of Erk1/2 pathway favors neutrophil over monocyte/macrophage development in Lin− BM cells cultured in M-CSF. Thus, prolonged Erk1/2 activation results in monocyte development following G-CSF induction whereas inhibition of Erk1/2 signaling promotes neutrophil development at the expense of monocyte cell fate in response to M-CSF. It appears that the signals for terminal differentiation transduced by G-CSFR and M-CSFR might be similar, but the decision whether to develop along the neutrophil or monocytes/macrophage lineage largely depends on the duration and probably also the magnitude of Erk1/2 activation. These results point to an important mechanism by which G-CSF and M-CSF instruct neutrophil versus monocyte lineage choice, i.e. differential activation of Erk1/2 pathway. In support
of this, it has been shown that inhibition of Erk1/2 signaling with U0126 or through overexpression of the Erk1/2-specific nuclear phosphatase DUSP5 led to neutrophil instead of mono
cyte development in response to G-CSF in a mouse E2A-Pbx1-
immortalized pro-T cell line transfected with M-CSFR (42, 43).

The Erk1/2 signaling pathway has been shown to play a piv-
ottal role in cell differentiation in different cellular systems. For
instance, sustained ERK1/2 activation by nerve growth factor
(NGF) induces neuronal differentiation in PC12 cells (44, 45).
Thrombopoietin-induced megakaryocytic differentiation is de-
pendent on prolonged activation of the Ras-Erk1/2 pathway
(46). Persistent Erk1/2 signaling has also been shown to be
required for M-CSF-induced monocytic differentiation in mye-
loid FDCP1 cells (33) and that the Mek1/2 inhibitors U0126 and
PD98059 inhibit the production of monocytes/macrophages
from primary BM cells in vitro (32, 47). The data presented here
indicate that Erk1/2 pathway regulates neutrophil versus
monocyte lineage choice, but is not required for the process of
terminal differentiation. It is of note that treatment of Lin− BM
cells cultured in M-CSF with U0126 and PD0325901 not only
decreases the production of monocytes and macrophages, but
also increases the total number of neutrophils, indicating that
inhibition of Erk1/2 signaling redirects the Lin− BM cells to
develop along the alternative neutrophil lineage in response to
M-CSF. However, monocyte/macrophage development is not
completely blocked by the Mek1/2 inhibitors in Lin− BM cells.
It is possible that Erk1/2 pathway promotes, but is not essential
for monocyte development. An alternative explanation is that
some Lin− cells are already committed to the monocyte lineage
and their terminal differentiation is not dependent on Erk1/2
signaling.

Our data also indicate that the Erk1/2 pathway promotes
monocyte cell fate through c-Fos and Egr1. Prolonged Erk1/2
activation by G-CSF Y729F is associated with more sustained
induction, greater mobility shifts and augmented activation of
c-Fos and Egr1, consistent with the previous study demonstrat-
ing that the persistently activated Erk1/2 directly phosphory-
late and stabilize c-Fos and likely other early response gene
products including Egr1 (36, 37). Phosphorylation of c-Fos by
Erk1/2 also primes it for additional phosphorylation (37).
Indeed, G-CSF-induced c-Fos phosphorylation on Ser32 was
more sustained in cells expressing G-CSF Y729F than in cells
expressing WT G-CSFR. Egr1 has been shown to support
monopoiesis and suppresses the alternative neutrophil cell fate
(14, 38). c-Fos has also been suggested to be involved in mono-
poiesis (5, 15, 39). Significantly, similar to the effects of Erk1/2
knockdown and the Mek1/2 inhibitors, knockdown of c-Fos or
Egr1 largely restores neutrophil cell fate in response to G-CSF.
Thus, c-Fos and Egr1 represent the key transcription factors
that are differentially activated by G-CSF and M-CSF in an
Erk1/2-dependent manner to resolve neutrophil versus mono-
cyte cell fate. Additionally, our data reveal for the first time a
critical role of c-Fos in monopoiesis.

In addition to activating c-Fos and Egr1, Erk1/2 have been
shown to phosphorylate serine 21 of C/EBPα and thereby
inhibit its activity (48). However, we have observed no signifi-
cant difference in C/EBPα activity upon G-CSF stimulation of
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