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Macrophage differentiation of myeloid progenitor cells in response to M-CSF is regulated by the dual-specificity phosphatase DUSP5

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ABSTRACT

M-CSF regulates the production, survival, and function of monocytes and macrophages. The MAPKs ERK1/2 are key elements for signal integration downstream of the M-CSFR, and their sustained activation is essential for macrophage differentiation. In this study, we sought to isolate genes whose induction by M-CSF is dependent on persistent MAPK activation, thereby being possibly involved in the commitment of myeloid progenitors to macrophage differentiation. Following SSH between cDNA libraries from FD-Fms cells stimulated by M-CSF for 8 h in the presence or the absence of the MEK inhibitor U0126, we isolated DUSP5. DUSP5 expression is induced by M-CSF in various myeloid cells and acts as a specific negative-feedback regulator of ERK1/2. In FD-Fms cells that proliferate and differentiate toward macrophages in response to M-CSF, overexpression of DUSP5 increased M-CSF-dependent proliferation and strongly decreased differentiation. Similarly, overexpression of DUSP5 in the multipotent EGER-Fms cells not only significantly increased M-CSF-induced proliferation and prevented macrophage differentiation but also favored granulocytic differentiation. Altogether, experiments demonstrated that DUSP5 is implicated in M-CSF signaling and suggested that it may influence myeloid cell fate. *J. Leukoc. Biol.* 87: 127–135; 2010.

Introduction

M-CSF or CSF-1 stimulates survival, proliferation, and differentiation of BM myelomonocytic progenitors, leading to the production of blood monocytes and tissue macrophages [1, 2]. A marked decline of osteoclast and macrophage populations results from M-CSF deficiency in osteopetrosis mice or targeted disruption of the mouse M-CSFR (or Fms) gene, unveiling the essential role of M-CSF in the development of the mononuclear phagocyte lineage [3, 4]. However, despite their overall similarity, several phenotypic characteristics of the mice defective in M-CSFR are more severe than those of the osteopetrosis mice [4]. The recent identification of IL-34 as a second ligand for the M-CSFR might explain these differences [5]. M-CSFR is a tyrosine kinase receptor that mediates all of the biological effects of M-CSF. The binding of M-CSF induces M-CSFR dimerization and its autophosphorylation on specific tyrosines of the cytoplasmic domain, creating binding sites for Src-homology 2-containing proteins, which in turn initiate multiple intracellular signaling pathways that cooperate to regulate gene expression and ultimately, cell survival, proliferation, and differentiation [6–8].

The Ras/MAPK signaling pathway is a key signal integration step inside of the cell for various extracellular stimuli, including M-CSF, and thus, represents a crucial determinant of biological outcome in a wide variety of tissues [9], including hematopoietic cells [10]. In macrophages, M-CSF activates the PI3K/Akt pathway and MAPK pathways, including ERK, p38, and JNK [11]. Earlier studies have enlightened the importance of a persistent activation of MAPK, resulting in transcription factor phosphorylation and selective activation of gene transcription [12, 13]. Similarly, we and others [14–16] have reported that the decision of myeloid progenitor cells to commit to macrophage differentiation in response to M-CSF also depends on activation levels of the MAPK, ERK1/2.

Abbreviations: ADAM8=a disintegrin and metalloproteinase 8, BMMP=bone marrow-derived macrophage precursor(s), CM=conditioned medium, DUSP5=dual-specificity phosphatase 5, FDC-P1=factor-dependent cell progenitor 1, FL=FLT 3 ligand, FSC=forward-scatter, GAP1^{m}=member of Ras GTPase-activating protein family, HPRT=hypoxanthine guanine phosphoribosyl transferase, MCL=mantle cell lymphoma, MGG=May-Grünwald-Giemsa reagent, MKP=MAPK phosphatase, OPN=osteopontin, p-ERK=phosphorylated ERK, PI=propidium iodide, qPCR=quantitative PCR, SCF=stem cell factor, SSC=side-scatter, SSH=suppressive subtractive hybridization, WT=wild-type

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For our studies, we used as a model the murine myeloid progenitor cell line FDC-P1, genetically engineered to express the murine M-CSFR (FD-Fms cells); these cells correspond to myeloid progenitors that self-renew in the presence of IL-3, whereas in response to M-CSF, their growth rate decreases, and cells acquire macrophage morphology [17]. In these cells, M-CSF induces late and persistent activity of ERK1/2, and inhibition of their phosphorylation with the MEK inhibitor U0126 prevents macrophage differentiation in response to M-CSF [15]. In an attempt to isolate M-CSF-responsive genes whose expression is also dependent on ERK1/2 activation, we have developed here a differential cDNA library-screening strategy based on a SSH technique that is sensitive to rare transcripts [18]. This led us to isolate several M-CSF-induced genes, among which we focused on DUSP5.

DUSPs constitute an important family of proteins that modulate spatiotemporal aspects of MAPK signaling [19–22]. DUSP5 (also known as hVH-3 or B23) is a nuclear MKP that dephosphorylates ERK1/2, not ERK5, p38 MAPK, and JNK, through specific and direct interaction [23]. The DUSP5 gene is induced by heat shock and growth factors [24–28] and is a direct target of p53 [29]. Herein, we provide evidence that DUSP5 is transiently induced by M-CSF in several myeloid progenitor cell types, which prompted us to study the effects of prolonged DUSP5 expression on macrophage differentiation. DUSP5 overexpression in FD-Fms cells decreased sustained ERK1/2 phosphorylation induced by M-CSF and inhibited the M-CSF differentiation signal. Moreover, in multipotent EGER-Fms cells, enforced expression of DUSP5 increased cell growth significantly and favored granulocyte versus monocyte differentiation. Taken together, these studies demonstrated that DUSP5 is able to regulate M-CSF signaling, and they strongly suggested that it may modulate the balance between proliferation and lineage commitment in myeloid progenitor cells.

MATERIALS AND METHODS

Cells

FDC-P1 cells expressing murine WT M-CSFR (FD-Fms cells) [17] were maintained in IMDM (Gibco, Cergy Pontoise, France), supplemented with 5% FBS (Dutscher, Brumath, France) and 5% of X63-IL-3-CM as a source of IL-3. For growth curves or macrophage differentiation assays, FD-Fms cells were washed free of growth factors, and 10^5 cells/ml were resuspended in IMDM-5% FBS, supplemented with 5% X63-IL3-CM or 2500 U/ml murine M-CSF secreted by High Five cells infected with recombinant baculovirus vector expressing M-CSF cDNA [30]. EGER-Fms cells [31] were maintained in OptiMEM (Gibco), supplemented with 10% FBS, 50 μM β-ME, 5% of BHK/MKL-CM as a source of SCF, 10 ng/ml murine rIL-7 (PeproTech, Levallois-Perret, France), and β-estradiol (1 μM; Sigma Chemical Co., St, Louis, MO, USA). For growth curves or macrophage differentiation assays, EGER-Fms cells were washed free of growth factors and β-estradiol and resuspended in OptiMEM-10% FBS-50 μM β-ME, supplemented with 5% BHK/MKL-CM, IL-7, and β-estradiol (IS+ condition) or only 2500 U/ml M-CSF (M– condition). Cells were examined daily and subcultured when they reached a density of 5×10^5 cells/ml. Murine BMMP were nonadherent cells obtained after cultivating BM cells from C57BL/6 mice for 6 days in IMDM-15% FBS, supplemented with 5 ng/ml human FL [32]. Macrophage differentiation of BMMP was obtained within 4 days after shifting the cells to IMDM-15% FBS, supplemented with 1000 U/ml M-CSF. The retrovirus packaging cell line Plat-E [33] was maintained in IMDM-5% FBS, supplemented with puromycin (1 μg/ml) and blasticidin (10 μg/ml).

Retroviral vectors and infections

*Hin*dIII-*Bgl*II fragments from cDNA of human WT DUSP5 or inactive C263S mutant (C/S) of DUSP5 [23, 26] were generated by PCR with a forward primer having the sequence 5′-GAGAGAAAGCTTAAGGTCACGTCGCTC-GAC-3′ and a reverse primer having the sequence 5′-GAGAGAGAGATCTCCG-CAGGATGTGGCCGT-3′ (restriction sites are underlined). The corresponding cDNAs were inserted in the *Hin*dIII-*Bam*HI restriction sites of pL(Flag-X-GF-P)SH retroviral vector [34] in frame with 5′-Flag and 3′-GFP sequences. Empty vector, pL(Flag-DUSP5 WT-GFP)SH, or pL(Flag-DUSP5 C/S-GFP)SH vectors were transfected in Plat-E cells. FD-Fms and EGER-Fms cells were infected for 1 day by coculture with these Plat-E virus-producing cells in the presence of 4 μg/ml polybrene (Sigma Chemical Co.) and selected for 1 week in the presence of hygromycin (1 mg/ml).

SSH

FD-Fms cells were starved free of growth factors for 3 h and then stimulated by M-CSF (2500 U/ml) for 8 h in the presence of 10 μM of U0126 (Calbiochem, San Diego, CA, USA) or 0.1% of DMSO. Total RNA was extracted using the RNeasy kit (Qiagen, Courtaboeuf, France) and subjected to DNase I treatment to avoid any genomic DNA contamination. Poly A+ RNA was extracted from total RNA using the Oligotex mRNA kit (Qiagen). Double-stranded cDNA synthesis and subtractive hybridization were performed by using the BD Clontech PCR-select cDNA subtraction kit (BD Biosciences, Le Pont-de-Claix, France), as specified by the manufacturer. Following the first round of subtraction, a subsequent PCR amplification of differentially expressed genes was performed, using the adaptor-specific primers 1 and 2R. PCR products were then cloned into a pCR4-TOPO vector (Invitrogen, Cergy Pontoise, France), sequenced using T7 primer, and sequences were compared with GenBank database using BLASTN algorithm.

RT-PCR and qPCR analyses

Total RNA was extracted as described above, and 2 μg was reverse-transcribed using random hexamers and the Omniscript RT kit (Qiagen). One-twentieth of the first-strand cDNA synthesis product was used as template for the PCR reactions using the Eppendorf Master Mix. Primers used were specific of murine OPN (sense, 5′-GGACCTCACCTCTCACATGAAGAGC-3′; antisense, 5′-CTAGTTGACCTCAGAAGATGAACTC-3′), murine ADAM8 (sense, 5′-TGGAG-GAGTTCTGTGATGGC-3′; antisense, 5′-ATTTGTCTCGGAGCCTTTCG-3′), murine GAP1^m (sense, 5′-CAGATTATTGGAAACTGGGG-3′; antisense, 5′-GT-GTGCTTTCACTCGTCTCC-3′), murine DUSP5 (sense, 5′-GAGTGCGTCT-GAGATTCTGTCCAG-3′; antisense 5′-AAGTCCAAGGTCACCGAGGAAC-3′), and murine HPRT (sense, 5′-CCCAGCGTCGTGATTAGCGATGATG-3′; antisense, 5′-CAAATCCAACAAAGTCTGGCCTGTATCC-3′). The thermal cycling program was 95°C for 30 s, followed by a hybridization step at 55°C for 30 s, and an elongation step at 72°C for 60 s. PCR products were size-fractionated on a 1.5% ethidium bromide-containing agarose gel. For qPCR, reactions were carried out with a Light Cycler 480 using SYBR Green PCR MasterMix (Roche Applied Science, Meylan, France). Expression values were normalized to the expression of the 18S rRNA: sense, 5′-CGGCTACCACATCCAAGGAA-3′; antisense, 5′-GCTGGAATTACCGCGGCT-3′.

Western blotting

Cells were washed with PBS and lysed in cold radio immunoprecipitation assay buffer (1% deoxycholic acid, 1% Triton X-100, 0.1% SDS, 50 mM Tris-base, 150 mM NaCl, 20 mM EDTA, pH 7.4) with the addition of a protease inhibitor cocktail (Roche Applied Science) and 2 mM Na_3VO_4 (Sigma Chemical Co.). Insoluble material was removed by centrifugation, and the protein concentration was determined by the Protein Assay solution (Bio-Rad, Hercules, CA, USA). Proteins from equalized cell lysates were separated on a SDS-polyacrylamide gel, transferred to nitrocellulose

membrane, and blotted with various antibodies as described previously [35]. Antibody binding was visualized using HRP-conjugated secondary antibodies (Sigma Chemical Co.) and ECL reagent (ECL+, GE Healthcare, Saclay, France). Antibodies used were anti-GFP (Roche Applied Science, Ref. 11814460001), anti-p-ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA, Ref. sc-7383), and anti-actin (Chemicon, El Segundo, CA, USA, MAB1501R).

Morphology and flow cytometry analyses

Cells were cytocentrifuged onto glass slides, air-dried, and stained with MGG (Sigma Chemical Co.). For flow cytometry determination of cell morphology, cells were washed with PBS and incubated 5 min in PBS-5% FBS containing 10 μg/ml PI (Sigma Chemical Co.). Samples were analyzed directly for light-scatter intensity to monitor cell size (FSC) and cell granularity (SSC) after exclusion of cells labeled by PI. For cell-surface antigen-expression analysis, cells were labeled by direct immunofluorescence, according to instructions from antibody manufacturers. PE-conjugated anti-Ly-6G and PE-Cy5-conjugated anti-CD11b antibodies were from Miltenyi Biotec (Germany) and Invitrogen, respectively. Before antibody labeling, cells were incubated for 10 min with anti-CD16/32 mAb (Invitrogen) to block FcRs. Cells were washed once in PBS before fluorescence analysis on a FACSCanto (Becton Dickinson, Le Pont-de-Claix, France).

Statistics

Results were expressed as the mean ± SEM for at least three independent experiments. Statistical significance was determined by Student's *t*-test (paired-data analysis). *P* values <0.05 were considered as statistically significant.

RESULTS

Identification of new M-CSF-induced genes

Experiments involving delayed addition of the MEK inhibitor U0126 indicated that FD-Fms cell differentiation requires maximal ERK1/2 activation between 8 and 24 h of M-CSF stimulation [15, 16]. Furthermore, we found that several genes associated with macrophage differentiation, including *Mona/Gads*, *Ifi203*, *Ifi204*, or *Egr-1*, were induced during that period of time [35, 36]. Therefore, we sought M-CSF-induced genes, whose expression is dependent on ERK1/2 activity, with the hope of identifying new genes implicated in the earliest steps of myeloid progenitor cell differentiation. For that purpose, SSH was used to compare mRNA populations from FD-Fms cells stimulated by M-CSF for 8 h in the absence or in the presence of U0126 inhibitor. By PCR amplification of differentially expressed sequences, we cloned 27 nonredundant sequences that might represent genes whose expression is increased by M-CSF treatment in a MEK-dependent manner. Among these candidate genes, RT-PCR analyses identified four genes exhibiting early and sustained induction in response to M-CSF, which could be abrogated by U0126: OPN, ADAM8, GAP1^m, and DUSP5 (**Fig. 1A**).

OPN and ADAM8 are known to be expressed during macrophage differentiation [37–40]. Interestingly, the two other isolated genes are negative regulators of MAPK signaling: GAP1^m [41] and DUSP5, an inducible nuclear MKP [23, 26]. Therefore, it was intriguing to find that M-CSF could induce negative regulators of the MAPK signaling pathway and control a cellular process that requires sustained activation of this same pathway. As neither DUSP5 expression nor its physiological

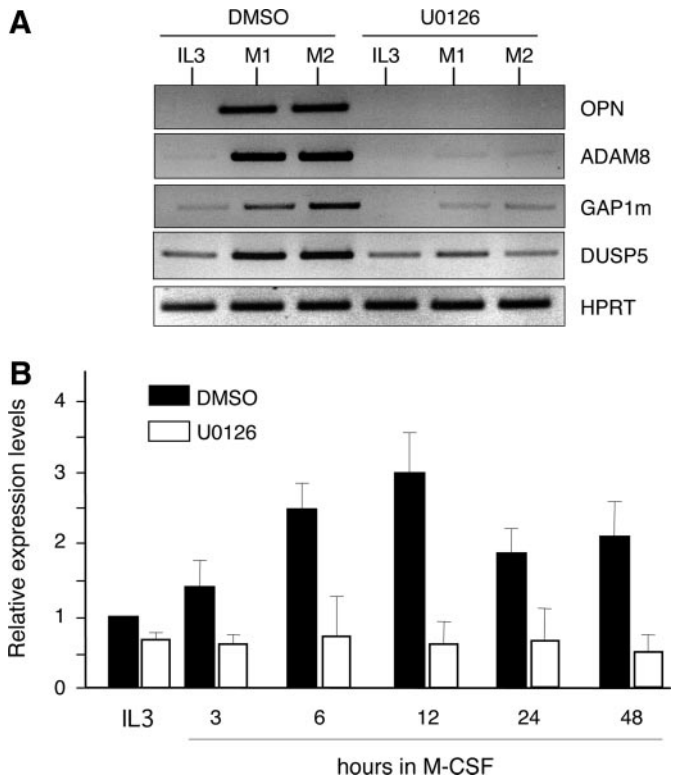

Figure 1. RT-PCR analysis of selected gene expression. (A) Expression of OPN, ADAM 8, GAP1^m, and DUSP5 transcripts was analyzed by RT-PCR using total RNA isolated from FD-Fms cells maintained in IL-3 or shifted to M-CSF-containing medium for 1 (M1) or 2 days (M2), in the absence or the presence of U0126 (10 μM). HPRT transcript expression was used as a control. (B) DUSP5 expression was analyzed by qRT-PCR, and the expression values were normalized to the expression levels of the 18S rRNA and expressed as fold differences versus the control (IL-3+DMSO); total RNA was extracted from FD-Fms cells maintained in the presence of IL-3 or shifted to M-CSF for different times in the absence (solid bars) or the presence of U0126 (10 μM; open bars). Data represent mean ± SEM for three independent experiments.

role has been evaluated so far in the myelomonocytic lineage, we went further into the investigation of DUSP5 induction and its role during macrophage differentiation. Before that, we confirmed by real-time qRT-PCR that DUSP5 was induced rapidly by M-CSF in FD-Fms cells in a MEK-dependent manner (Fig. 1B). Interestingly, DUSP5 mRNA peaked after 12 h of M-CSF stimulation, and then expression levels decreased but remained higher than in cells maintained in IL-3 (Fig. 1B).

DUSP5 induction is transient during macrophage differentiation

To confirm DUSP5 involvement in the response of myeloid cells to M-CSF, we examined two other models of macrophage differentiation. First, we used a primary culture system that permits massive in vitro expansion of BMMP, which are obtained following cultivation of BM cells in the presence of FL for 6 days, and then shifted to M-CSF-containing medium for progressive differentiation toward macrophages within 4 days

[32]. After 1 day of culture in the presence of M-CSF, DUSP5 expression was increased with a maximum after 2 days, and then DUSP5 expression decreased, and after 4 days of culture in the presence of M-CSF, i.e., at completion of macrophage differentiation, DUSP5 expression was similar to its initial level (**Fig. 2A**). As another model for macrophage differentiation, we used EGER-Fms cells, which represent a multipotent hematopoietic cell line immortalized by an estradiol-regulated version of E2a/Pbx1 oncoprotein. EGER-Fms cells self-renew in the presence of IL-7, SCF, and β-estradiol (IS+ medium), whereas they proliferate and differentiate into macrophages in the presence of M-CSF and the absence of β-estradiol (M– medium) [31]. As compared with FD-Fms cells, EGER-Fms cells require a longer period of time (8–10 days) to fully differentiate into growth-arrested macrophages in response to M-CSF, which is typical of normal myeloid progenitor differentiation [31]. After 1 day in the presence of M-CSF, DUSP5 expression increased approximately threefold and remained elevated for 1 more day and then declined after 3 days and returned to near baseline after 5 days of culture in the presence of M-CSF (Fig. 2B). It is to note that DUSP5 expression returned to its initial level when cells were washed free of M-CSF after 1 day of culture in M– medium and then resuspended in IS+ medium for another day (**Supplemental Fig. 1**), demonstrating that DUSP5 expression required the continuous presence of M-CSF in the culture medium. Altogether, previous experiments confirmed the rapid induction of DUSP5 in myeloid cell lines and native progenitor cells stimulated by M-CSF. Moreover, data obtained with BMMP and EGER-Fms cells showed that DUSP5 expression is transient and seems to be associated to early stages of differentiation, possibly commitment of myeloid progenitors.

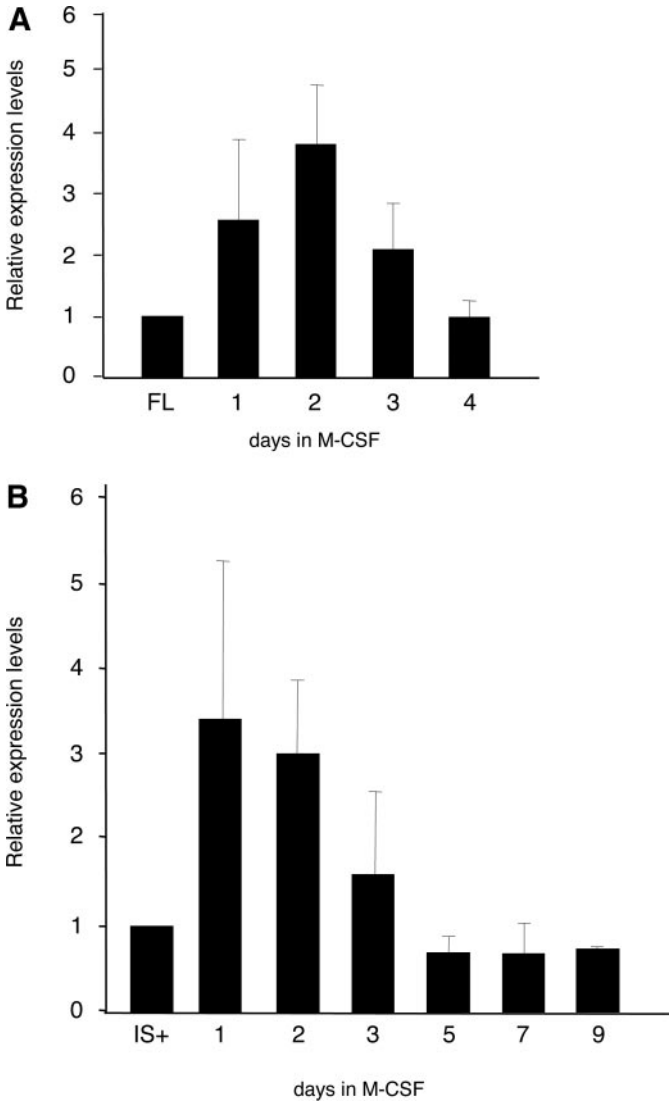

Figure 2. M-CSF transiently induces DUSP5 transcription in myeloid cells. DUSP5 expression was analyzed by qRT-PCR, and the expression values were normalized to the expression levels of the 18S rRNA and expressed as fold differences versus the control (FL or IS+); total RNA was extracted from: (A) BMMP expanded in the presence of FL or shifted to M-CSF-containing medium for 1, 2, 3, or 4 days; (B) EGER-Fms cells maintained in the presence of IL-7, SCF, and estradiol (IS+) or shifted to M-CSF-containing medium in the absence of estradiol for 1, 2, 3, 5, 7, or 9 days. Data represent mean ± SEM for three independent experiments.

DUSP5 activity regulates phosphorylation of ERK1/2 induced by M-CSF

To evaluate its possible role in the regulation of M-CSF-induced MAPK activity, DUSP5 protein was stably expressed in FD-Fms cells in the form of WT or a catalytically inactive mutant (C/S), in which the essential catalytic cysteine residue (C263) was mutated to a serine residue [23, 26]. FD-Fms cells were retrovirally transduced with vectors encoding or not human WT or C/S DUSP5 fused to the GFP protein. Infected FD-Fms cells were selected for hygromycin resistance and expanded in IL-3. FACS and Western blot analyses showed that selected cells expressed a significant amount of GFP proteins and that fusion proteins were detected at the expected size of ~70 kDa (**Fig. 3A**). For each population, three clones exhibiting representative expression of GFP were selected and used in parallel throughout the study about the FD-Fms cell line. Crude molecular (**Figs.** 3B and **4C**) and cellular (Fig. 4A) data are shown for a single clone/population (LX-c7 for control, WT-c15 for WT DUSP5, and C/S-c24 for C/S DUSP5), whereas statistics about differentiation (Fig. 4B) and proliferation (Fig. 4D) were established by pooling data from three independent experiments performed on the three clones. To evaluate the effects of overexpressed DUSP5 on MAPK phosphorylation, we compared levels of ERK1/2 phosphorylation in FD-Fms cells cultivated under conditions of self-renewal (IL-3) or differentiation (M-CSF for 1 or 2 days). In control LX-c7 cells, ERK1/2 phosphorylation was higher in the presence of M-CSF than in the presence of IL-3, with a peak after 1 day (Fig. 3B). In WT-c15 cells expressing WT DUSP5, phosphorylation of ERK1/2 was decreased severely after 1 day in M-CSF as compared with control LX-c7 cells in the same condition and even undetectable after 2 days in the presence of M-CSF (Fig. 3B). Thus, it was concluded that DUSP5 might negatively regulate M-CSF-dependent ERK1/2 activation in FD-Fms cells. Furthermore, cells expressing the inactive form of DUSP5 (C/S-c24) showed markedly increased ERK1/2

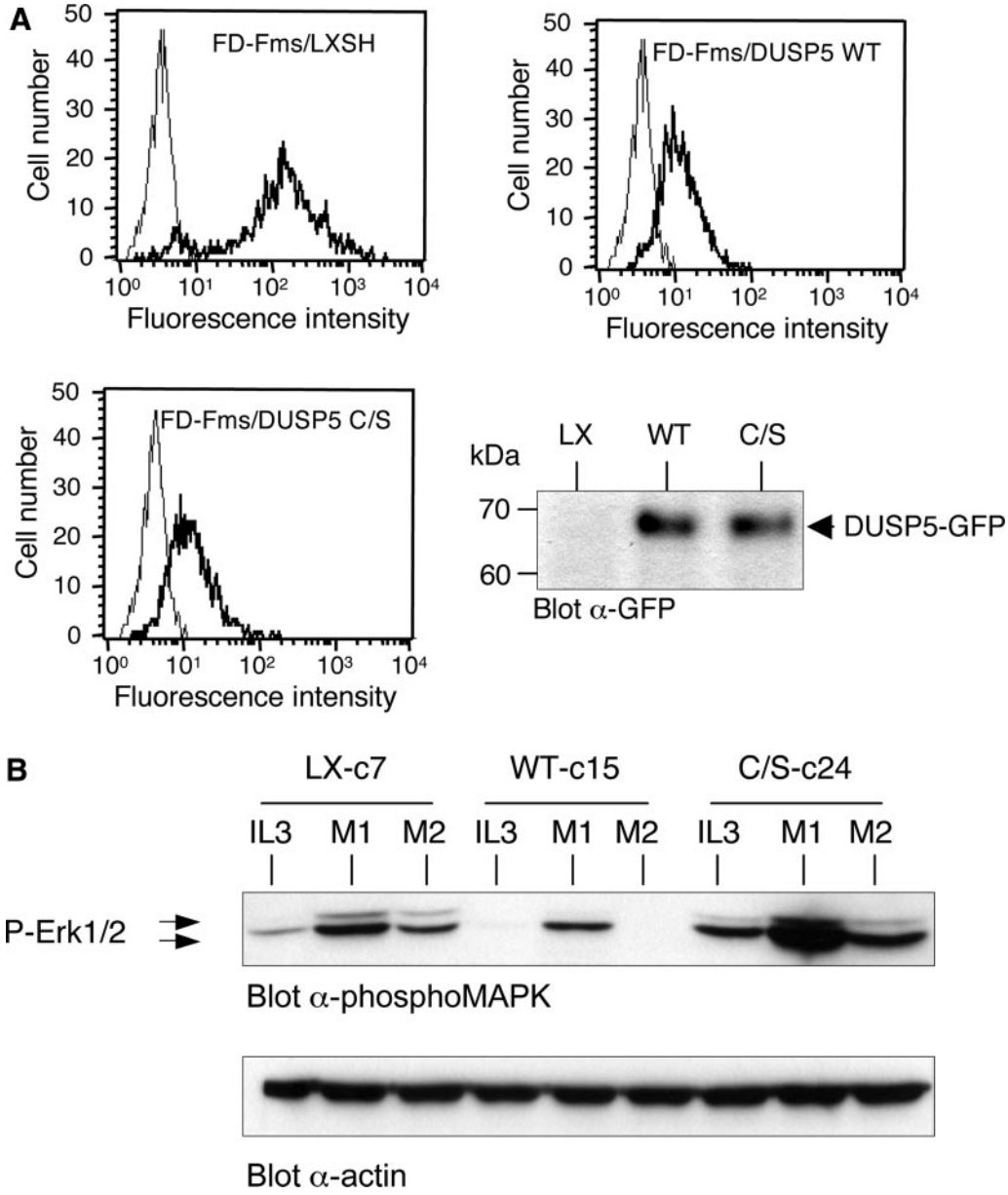

Figure 3. DUSP5 regulates prolonged and increased ERK1/2 phosphorylation in response to M-CSF. (A) Flow cytometry and Western blot analysis of GFP expression after hygromycin selection of FD-Fms cells infected with a retroviral vector that enables expression of a fusion protein between DUSP5 WT or mutated (C/S) and GFP; FD-Fms cells infected with the empty vector expressed only GFP; noninfected FD-Fms cells were used as a negative control. (B) Representative clones of each FD-Fms cell population containing the empty retroviral vector (LX-c7), WT DUSP5 (WT-c15), or mutated DUSP5 (C/S-c24) were cultivated in IL-3 or shifted to M-CSF-containing medium for 1 or 2 days. Then, cells were lysed, and equal amounts of whole-cell lysates (40 μg) were analyzed by SDS-PAGE and Western blotting using anti-p-ERK1/2 antibody; the blot was stripped and reprobed for the ubiquitous actin protein as a loading control. Data are representative of three independent experiments.

phosphorylation when compared with LX-c7 cells, irrespective of culture conditions (Fig. 3B). This suggests a dominant-negative effect of the mutant protein on endogenous DUSP5, as described previously in T lymphocytes [26]. DUSP5 phosphatase activity is considered to be specific of ERK1/2 [23], but DUSP5 overexpression may affect other MAPKs directly or indirectly modulate other signaling pathways implicated downstream of M-CSFR or in macrophage differentiation. For these reasons, we verified any possible effect of DUSP5 overexpression on AKT [42] or JNK [43] phosphorylations. AKT phosphorylation was increased after 1 day in the presence of M-CSF, and no difference was observed among the three cell populations, irrespective of the culture conditions (data not shown). Concerning JNK, no phosphorylation was detected in all three populations, suggesting that in FD-Fms cells, M-CSF did not activate this signaling pathway (data not shown). Altogether, these results strengthened the idea that DUSP5 specifically inactivate the ERK1/2 MAPK in myeloid FD-Fms cells, as described previously in fibroblasts and T lymphocytes [23].

DUSP5 activity regulates macrophage differentiation induced by M-CSF

M-CSF-stimulated differentiation requires persistent MEK activity and subsequent ERK1/2 activation [15, 16, 44]. As shown above, overexpressed DUSP5 is able to prevent increased and prolonged ERK1/2 phosphorylation in response to M-CSF. Therefore, we asked if DUSP5 would also alter macrophage differentiation of FD-Fms cells. Like parental cells [15–17], control LX-c7 cells exhibited a blast morphology and low cellular granularity in the presence of IL-3, whereas they acquired macrophage morphology and increased cellular granularity after 2 days in the presence of

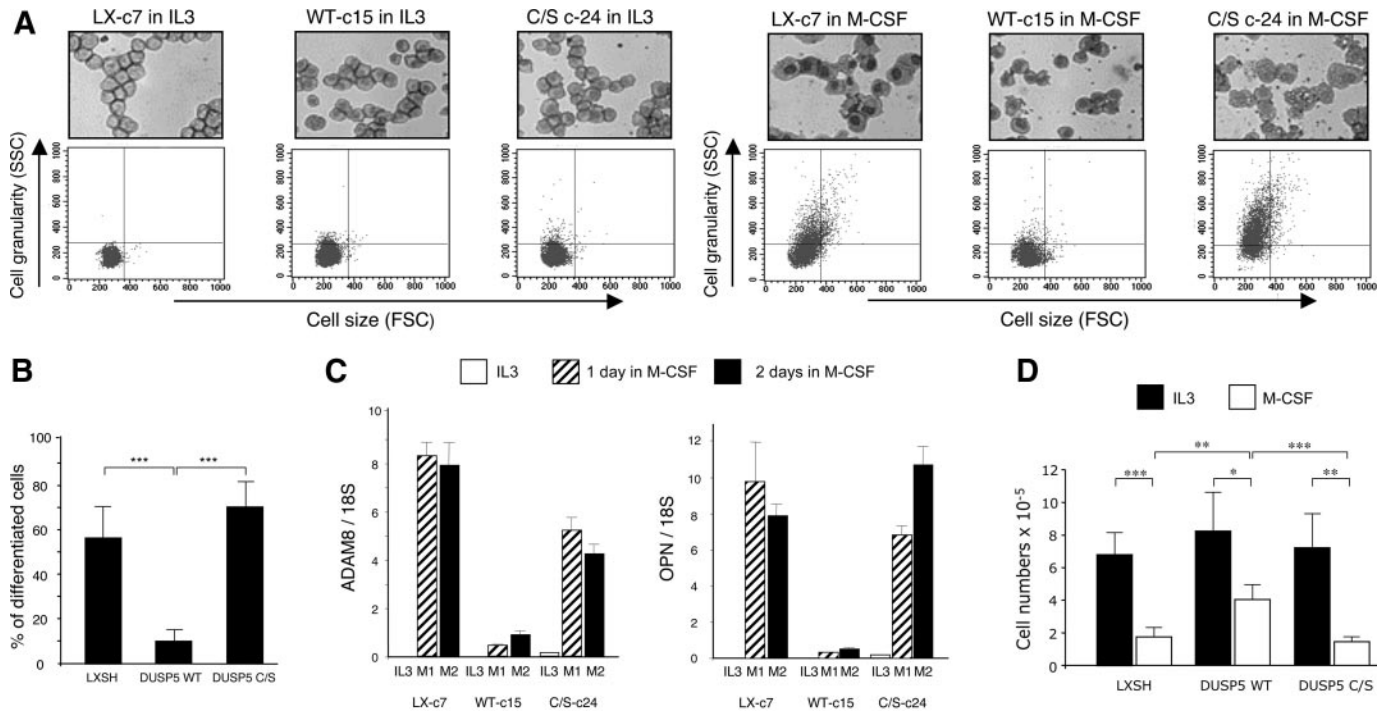

Figure 4. Enforced expression of WT DUSP5 alters M-CSF-induced macrophage differentiation of FD-Fms cells. (A) Clones representative of each FD-Fms cell population were maintained in IL-3 or shifted to M-CSF-containing medium for 2 days, and cell morphology was examined after staining with MGG reagent or by flow cytometry for cellular size (FSC) and granularity (SSC; dot diagrams). (B) For each FD-Fms cell population, three different clones were cultivated in IL-3 or in M-CSF for 2 days and then analyzed by flow cytometry; percentage of differentiated cells corresponds to cells with a higher granularity than undifferentiated control cells maintained in the presence of IL-3; data are mean ± SEM for three independent experiments. (C) ADAM8 (left panel) and OPN (right panel) transcript expression was analyzed by qRT-PCR using total RNA isolated from one representative clone of each FD-Fms cell population, maintained in IL-3 (open bars) or shifted to M-CSF for 1 day (hatched bars) or 2 days (solid bars); the expression values were normalized to the expression levels of the 18S rRNA. Data represent mean ± SEM for three independent experiments. (D) Three clones of each FD-Fms cell population (10^5 cells/ml) were cultivated in liquid cultures supplemented with IL-3 (solid bars) or M-CSF (open bars); after 2 days, cells were scored. Data represent mean ± SEM for three independent experiments. (B and D) *P* values: *, $P < 10^{-3}$; **, $P < 10^{-4}$; ***, $P < 10^{-5}$.

M-CSF (Fig. 4A). In contrast, WT-C15 cells exhibited only a slight modification of their morphology after 2 days in the presence of M-CSF (Fig. 4A), and only 10.1 ± 6.1% of FD-Fms/DUSP5 WT cells exhibited an increased cellular granularity as compared with 56.7 ± 14.0% for control FD-Fms/LXSH cells (Fig. 4B). Differentiation block was not observed with cells expressing the inactive form of DUSP5 (Fig. 4A, C/S-c-24), and 70.4 ± 11.7% of FD-Fms/DUSP5 C/S cells had an increased cellular granularity (Fig. 4B). Consistent with morphological analyses, M-CSF induction of the differentiation markers ADAM8 and OPN was decreased dramatically in WT-c15 cells as compared with LX-c7 or C/S-c24 cells (Fig. 4C).

Finally, M-CSF-dependent proliferation of the different clones was examined, as macrophage differentiation is associated with slowed-down growth [17]. We observed that there was a significant twofold growth increase in the presence of M-CSF with FD-Fms/DUSP5 WT cells as compared with control FD-Fms/LXSH or FD-Fms/DUSP5 C/S cells (Fig. 4D). In conclusion, we showed that DUSP5 expression is favoring growth over differentiation of FD-Fms cells in response to M-CSF.

DUSP5 favors commitment to granulocytic differentiation in EGER-Fms cells

DUSP5 was transiently induced by M-CSF in EGER-Fms cells (Fig. 2B). As MEK inhibition converts the M-CSF differentiation signal from a macrophage to granulocyte pathway in these cells [31], we examined proliferation and differentiation of EGER-Fms cells expressing WT DUSP5 or C/S DUSP5 in response to M-CSF. Expression of either DUSP5 form did not affect their proliferation rate under self-renewal culture conditions (**Fig. 5A**, IS+, left panel). In response to M-CSF, control EGER-Fms/LXSH cells first proliferated, but growth rate progressively decreased, and cells stopped growing after day 8 (Fig. 5A, M–, right panel). Morphology examination after 9 days in the presence of M-CSF showed that cells had differentiated into monocytes/macrophages (Fig. 5B, upper left panel), as described previously [31]. A similar response to M-CSF was obtained with EGER-Fms cells expressing inactive C/S DUSP5 (Fig. 5, A, M–, right panel, and B, upper right panel). In contrast, EGER-Fms cells expressing WT DUSP5, cultivated in the presence of M-CSF, exhibited a much stronger proliferation rate than the two other cell populations, before cells stopped

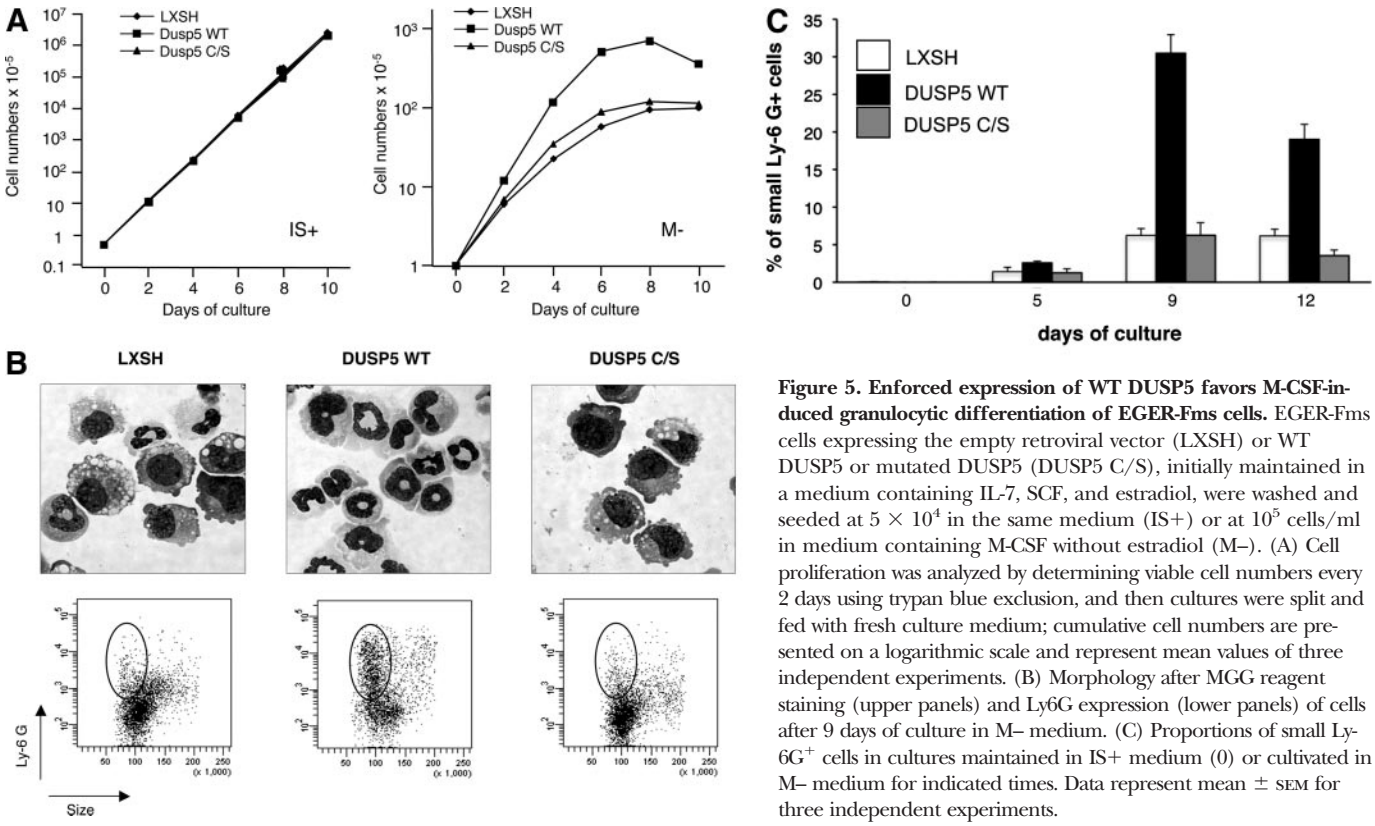

Figure 5. Enforced expression of WT DUSP5 favors M-CSF-induced granulocytic differentiation of EGER-Fms cells. EGER-Fms cells expressing the empty retroviral vector (LXSH) or WT DUSP5 or mutated DUSP5 (DUSP5 C/S), initially maintained in a medium containing IL-7, SCF, and estradiol, were washed and seeded at 5×10^4 in the same medium (IS+) or at 10^5 cells/ml in medium containing M-CSF without estradiol (M–). (A) Cell proliferation was analyzed by determining viable cell numbers every 2 days using trypan blue exclusion, and then cultures were split and fed with fresh culture medium; cumulative cell numbers are presented on a logarithmic scale and represent mean values of three independent experiments. (B) Morphology after MGG reagent staining (upper panels) and Ly6G expression (lower panels) of cells after 9 days of culture in M– medium. (C) Proportions of small Ly-$6G^+$ cells in cultures maintained in IS+ medium (0) or cultivated in M– medium for indicated times. Data represent mean ± SEM for three independent experiments.

growing after 1 week (Fig. 5A, M–, right panel). At that time, the majority of the cells exhibited a granulocytic morphology (Fig. 5B, upper middle panel), which we sought to verify by flow cytometry characterization of Ly-6G, a selective granulocyte marker in the mouse bone marrow [45].

After 9 days of culture, in the presence of M-CSF, EGER-Fms/DUSP5 WT cells, but not EGER-Fms/LXSH or EGER-Fms/DUSP5 C/S cells, showed a distinct population of cells displaying small size and strong expression of Ly-6G (Fig. 5B, lower panels). These cells likely correspond to the mature granulocytic cells seen in MGG-stained preparations. Follow-up of small Ly-$6G^+$ cells in M-CSF-stimulated cultures confirmed weak granulocytic differentiation in EGER-Fms/LXSH and EGER-Fms/DUSP5 C/S cells and the remarkably strong induction of Ly-6G in EGER-Fms/DUSP5 WT cells (Fig. 5C). Meanwhile, kinetics of CD11b expression in M-CSF-stimulated cultures was similar in the three cell lines, confirming their myeloid differentiation potential (**Supplemental Fig. 2**). Altogether, data indicated that overexpression of WT DUSP5 reduced monocytic and favored granulocytic development of EGER-Fms cells in response to M-CSF, suggesting that DUSP5 might act as a mediator for progenitor myeloid cell-fate determination.

DISCUSSION

In this study, we sought to isolate genes induced in myeloid cells in response to M-CSF through the MAPK/ERK pathway. Four new M-CSF-responsive genes have then been isolated using the SSH technique. Two of them were already known to be expressed in the monocytic lineage: *OPN* gene encodes OPN, a heavily glycosylated phosphoprotein implicated in macrophage function and differentiation [37, 38]; *ADAM8* gene encodes a transmembrane glycoprotein with proteolytic activity implicated in cell adhesion, cell fusion, and cell signaling [39, 40]. Recently, higher expression of ADAM8 has been reported in a subset of human blood monocytes, suggesting a specific function for ADAM8 in these cells [46]; ADAM8 knockout mice [47] would offer a valuable tool to investigate ADAM8 functions in these M-CSF-responsive cells.

We have also isolated two key regulators of the Ras/MAPK signaling pathway. $GAP1^m$ is up-regulated in oncogenic ras-transformed fibroblasts [48]. $GAP1^m$ transcript expression is induced by growth factors and is dependent on MEK activity in transformed NIH 3T3 cells [48], which is consistent with our results. However, neither $GAP1^m$ expression nor its role during hematopoiesis has been documented so far. Finally, we decided to focus our study on DUSP5, a member of an important family of dual-specificity phosphatases that influence spatiotemporal aspects of ERK1/2 signaling [22, 23, 49, 50]. DUSP5 is an inducible nuclear protein that dephosphorylates and inactivates ERK1/2 specifically, and it was proposed that it could also function as a nuclear anchor for ERK2 [22]. In agreement with our results, it has been demonstrated recently that induction of DUSP5 mRNA in response to growth factors is dependent on ERK1/2 activation in fibroblasts [51]. Moreover, the accumulation of DUSP5 protein is regulated by rapid proteasomal degradation, and significant DUSP5 stabilization

was observed following its association with ERK1/2. Interestingly, DUSP5 is phosphorylated by ERK1/2 on three sites, although biological function of these modifications remains unknown [51].

DUSP5 expression and function have been studied extensively in T cell development; data clearly pointed toward potential roles for DUSP5 in various settings of lymphocyte development and activation [26, 27, 52]. Thus, T cell-targeted overexpression of DUSP5 in transgenic mice results in the block of thymocyte development at the double-positive stage, and transgenic mice develop autoimmune symptoms [50], confirming DUSP5 as a critical component of normal immune development. As compared with normal B cells, MCL, a non-Hodgkin lymphoma with poor prognosis, exhibits up-regulated DUSP5. Its knockdown using RNA interference induced growth arrest of MCL cells, strongly suggesting that DUPS5 is involved in regulation of lymphoma growth [53]. More recently, expression and function of DUSP5 in angioblasts have been unveiled, demonstrating that DUSP5 together with sucrose nonfermenting 1-related kinase 1 target a signaling pathway critical for embryonic vascular development [54]. Importantly, DUSP5 somatic mutations have been found in different types of vascular malformations and tumors [54]. Altogether, these results oppose the traditional view that phosphatases simply act to terminate or decrease active signaling and demonstrate that an MKP, like DUSP5, could tilt cell development by controlling proliferation, differentiation, and/or apoptosis.

To our knowledge, the present report is the first concerning DUSP5 expression and its function during myeloid development. DUSP5 was identified as a M-CSF-regulated gene in myeloid cells, including native BM-derived monocyte precursors. We have shown that DUSP5 can act as a negative-feedback regulator preventing sustained ERK1/2 activation in response to M-CSF. Given that strong and sustained activation of ERK1/2 is essential for M-CSF-induced macrophage differentiation [15, 16], the induction of a negative regulator of ERK1/2 activity seemed surprising. DUPS5 induction at early times of macrophage differentiation might occur to favor cellular expansion over terminal differentiation and subsequent growth arrest. Accordingly, when WT DUSP5 was overexpressed in FD-Fms cells, we observed an increased proliferation and a block of differentiation in response to M-CSF, whereas the enforced expression of an inactive mutant of DUSP5 had opposing effects.

In accordance with our results, it has been shown recently that several MKPs are induced by M-CSF in BM-derived macrophages with distinct patterns of induction; in these cells, IFN-γ inhibits the expression of some of these MKPs, leading to prolonged MAPK activity and inhibition of proliferation [55]. Interestingly, IFN-γ and M-CSF could also act synergistically to promote differentiation and growth arrest of FD-Fms cells [56], which may suggest that DUSP5 is also involved in this mechanism.

However, biological effects of DUSP5 depend on the cellular context, as in EGER-Fms cells, overexpression of DUSP5 not only significantly increased M-CSF-induced proliferation and prevented macrophage differentiation, but also, instead, EGER-Fms cells differentiated into granulocytes. This is consistent with our previous observation that EGER-Fms cells treated with an inhibitor of MEK undergo granulocytic differentiation in response to M-CSF [31]. Thus, DUSP5 can influence myeloid cell fate, probably by modulating the level of ERK1/2 activity. It is clear that ERK signaling is a molecular switch integrating multiple inputs from various cytokines, and we propose that DUSP5 expression may participate in the cell-lineage decision dictated to myeloid progenitors by their environment.

AUTHORSHIP

R. P. B. designed research, performed research, collected data, analyzed data, and wrote the paper; M-F. G, S. G-G., and G. M. designed research, performed research, collected data, and analyzed data. M-F. G. and S. G-G. contributed equally to this work.

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