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PLCγ2 and PKC Are Important to Myeloid Lineage Commitment Triggered by M-SCF and G-CSF

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ABSTRACT

Myeloid differentiation is a complex process whereby mature granulocytes or monocytes/macrophages are derived from a common myeloid progenitor through the coordinated action of hematopoietic cytokines. In this study, we explored the role of the $Ca^{2+}{}_i$ signaling transduction pathway in the commitment of hematopoietic stem/progenitor cells to either the monocytic or granulocytic lineage in response to macrophage colony-stimulating factor (M-CSF) and granulocyte colony-stimulating factor (G-CSF). M-CSF and G-CSF induce cell expansion and monocyte or granulocyte differentiation, respectively, without affecting the percentage of hematopoietic progenitor cells. Colony-forming units (CFUs) and flow cytometry demonstrated the involvement of phospholipase Cγ (PLCγ) and protein kinase C (PKC) in monocyte/granulocyte commitment. In addition, using flow cytometry and RNA interference, we identified PLCγ2 as the PLCγ isoform that participates in this cell expansion and differentiation. Differences in signaling elicited by M-CSF and G-CSF were observed. The M-CSF-related effects were associated with the activation of ERK1/2 and nuclear factor of activated T-cells (NFAT); the inhibition of both molecules reduced the number of colonies in a CFU assay. In contrast, using flow cytometry and confocal evaluation, we demonstrated that G-CSF activated Jak-1 and STAT-3. Additionally, the effects induced by G-CSF were also related with the participation of Ca^{2+} calmodulin kinase II and the transcription factor PU.1. STAT-3 activation and the increase of PU.1 expression were sensitive to PLC inhibition by U73122. These data show that PLCγ2 and PKC are important upstream signals that regulate myelopoiesis through cytokines, and differences in M-CSF and G-CSF downstream signaling were identified. J. Cell. Biochem. 115: 42–51, 2014. © 2013 Wiley Periodicals, Inc.

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Hematopoiesis is initiated by a rare multipotent population of bone marrow cells called hematopoietic stem cells (HSCs), which, at each cell division, must decide whether to self-renew, differentiate, migrate or die [Weissman, 2000]. HSCs can differentiate into common myeloid progenitors (CMPs) that then can produce granulocytes and monocytes/macrophages; this differentiation process is mainly regulated by hematopoietic myeloid cytokines [Weissman, 2000]. Cytokines control these processes by binding to specific cell-surface receptors in a stage-specific and lineage-specific manner, resulting in the activation of intracellular signal transduction pathways that are important for proliferation, survival, and differentiation [Miranda and Johnson, 2007].

Macrophage-colony stimulating factor (M-CSF) and granulocyte-colony stimulating factor (G-CSF) are cytokines that play key roles in

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promoting monocytic/macrophage and granulocytic differentiation, respectively. The binding of G-CSF to its cognate receptors has been shown to result in the activation of a number of intracellular signaling pathways, including the Jak/STAT, Src kinase, and Ras/Raf/MEK/ERK pathways [Ward et al., 2000]. The M-CSF receptor dimerizes upon interaction with M-CSF and promotes intrinsic tyrosine kinase activity, which in turn activates the Ras/Raf/MEK/ERK, phospholipase Cγ (PLCγ), phosphatidylinositol 3-kinase (PI3K), and Src kinases [Bourette and Rohrschneider, 2000]. These signaling proteins serve to activate myeloid transcription factors, such PU.1, C/EBP, IRF8, and Myc, which drive the differentiation process [Rosenbauer and Tenen, 2007].

Emerging evidence indicates that multiple signaling pathways, which sometimes play a redundant role in the physiological function of mature myeloid cells, are activated during cytokine-induced myeloid differentiation. One pathway whose role is not fully understood in hematopoiesis is the Ca^{2+} signaling pathway. This pathway has remarkably versatile signaling that controls many physiological processes, such as proliferation, fertilization, muscle contraction, exocytosis, and cell death, in all cell types [Berridge, 2009]. The Ca^{2+} pathway can participate in cytokine signaling through the activation of PLCγ, which recognizes the phosphorylation of intracellular tyrosine residues in activated cytokines receptors [Whetton et al., 1988; Ren et al., 1994; Collison et al., 1998; Tong et al., 2004; Paredes-Gamero et al., 2008; Leon et al., 2011]. The activation of PLCγ results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), thus forming inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) as second messengers [Berridge and Irvine, 1984]. IP_3 spreads into the cytoplasm and opens the IP_3 receptor channel, releasing intracellular Ca^{2+} ($Ca^{2+}{}_i$) from the endoplasmic reticulum, and DAG induces protein kinase C (PKC) activation. Moreover, $Ca^{2+}{}_i$ can couple with other proteins, such as Ca^{2+}-calmodulin (CaM), which is an activator of the Ca^{2+}/CaM dependent protein kinases (CaMK) [Cullen and Lockyer, 2002; Berridge, 2009].

Recently, we have demonstrated the direct involvement of Ca^{2+} signaling in the proliferation and differentiation of hematopoietic stem/progenitor cells. The transient increase of $Ca^{2+}{}_i$ induced by IL-3 and GM-CSF is related to cytokine-dependent proliferation, which is involved with the activation of Ca^{2+} signaling molecules such as PLCγ, PKC, and CaMKII [Paredes-Gamero et al., 2008]. In addition, we described that IL-3 and GM-CSF lead to the activation of PLCγ2, which induces IP_3 formation and the release of $Ca^{2+}{}_i$, which is followed by the activation of Ca^{2+}-dependent kinases that modulate the MEK/ERK1/2 pathway in hematopoietic stem/progenitor cells [Leon et al., 2011]. Moreover, other Ca^{2+} signaling molecules such as CaMKIV have been described as a regulators of HSC homeostasis; for example, $Camk4^{-/-}$ mice display enhanced proliferation, increased apoptosis, and decreased levels of phospho-CREB and Bcl-2 protein [Kitsos et al., 2005]. Furthermore, the participation of Ca^{2+} signaling in HSC differentiation by ATP has been demonstrated [Barbosa et al., 2011].

In this study, the role of Ca^{2+} signaling transduction was explored in myeloid proliferation and in the differentiation of hematopoietic stem/progenitors cells into monocytic and granulocytic lineages following stimulation with M-CSF and G-CSF; the integration of Ca^{2+} signaling with other intracellular pathways that regulate hematopoiesis was also examined.

MATERIALS AND METHODS

ANIMALS

The wild-type mice (C57BL/6, 8–12 weeks old) used in this study were supplied by the INFAR/UNIFESP Animal Facility (São Paulo, Brazil). All experiments were approved by the Animal Care and Ethics Committee of the Federal University of São Paulo (0254/08).

DEXTER-TYPE CULTURES

Long-term bone marrow cultures (LTBMCs) were prepared as previously described [Dexter et al., 1977] with some modifications. Briefly, total bone marrow cells were plated into 12-well plates and fed weekly with Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 5% FBS (Cultilab, Brazil), 20% horse serum (StemCell Technologies, Tukwila, WA) and 1 μM hydrocortisone (Sigma–Aldrich, St Louis, MO). The cultures were maintained at 37°C under 5% CO_2. After the establishment of a confluent stromal layer, the remaining non-adherent cells were removed, and 10^6 cells per well were added to the precultured stroma, along with fresh media. After 1 week of coculture, the cells were further cultured in IMDM with 0.5% FBS for 24 h. Subsequently, LTBMCs were stimulated with M-CSF or G-CSF. The cells were harvested after 24, 48, and 72 h and counted in a hemocytometer chamber. After 3 days, the cells were collected for immunophenotyping using the antibodies Gr-1-FITC, Mac-1-PE, and c-Kit-APC. The Gr-1^-Mac-1^-c-Kit^+ population was identified as a hematopoietic stem/progenitor population [Paredes-Gamero et al., 2008]. These data analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson, San Diego, CA). In addition, cocultured cells were stained with Giemsa/May-Grünwald solution (Sigma-Aldrich, St Louis, MO) and identified by morphological characteristics.

FLOW CYTOMETRY ANALYSIS

LTBMCs were incubated for 24 h in IMDM with 0.5% FBS, and then stimulated with M-CSF (10 ng/ml) or G-CSF (10 ng/ml) at 37°C under 5% CO_2. The cells were fixed with 2% paraformaldehyde (Becton Dickinson) for 30 min. The cells were washed with 0.1 M glycine and permeabilized with 0.01% saponin (phospho-PLCγ1 [p-PLCγ1_{Tyr783}], p-PLCγ2_{Tyr759}, p-CaMKII$_{thr286}$, p-PKC$_{Thr514}$, p-JAK-$1_{Tyr1022/1023}$, p-JAK-$2_{Tyr1008}$, p-AKT$_{Thr308}$) or 0.001% Triton (p-ERK$1/2_{Thr202/Thr204}$, p-JNK$_{Thr183/Tyr185}$, p-p$38_{Thr180/Tyr182}$, p-STAT3_{Tyr705}, p-STAT5_{Tyr694}, c-Jun, PU.1) for 15 min. Subsequently, the cells were incubated for 2 h with rabbit anti-p-PKC, anti-p-CaMKII, anti-p-PLCγ1, anti-p-PLCγ2, anti-p-AKT, anti-p-p38, anti-p-JNK, anti-p-Jak1, anti-p-Jak2, anti-p-ERK1/2, anti-p-STAT-3, anti-p-STAT-5, or anti-c-Jun antibodies (Cell Signaling Technology, Tukwila, WA). Next, the cells were incubated for 40 min with the appropriate rabbit anti-IgG-Alexa Fluor 488-conjugated secondary antibodies (Invitrogen, USA). All antibodies were diluted in PBS with 1% albumin. The transcription factor PU.1 was recognized using an anti-PU.1 Alexa Fluor 488-conjugated antibody (Becton Dickinson). The phosphorylation of proteins was evaluated in the Gr-1^-Mac-1^-c-Kit^+ population using

flow cytometry (FACSCalibur, Becton Dickinson), and the analysis was performed using the WinMDI 2.8 software.

CONFOCAL MICROSCOPY

Bone marrow cells were seeded on glass cover slides (13 mm) under the conditions described above. These cells were cultured in 0.5% FBS (24 h) and stimulated with G-CSF. Subsequently, the cells were fixed with 2% paraformaldehyde for 30 min, washed with 0.1 M glycine, permeabilized with 0.01% saponin for 15 min, and then washed with PBS. The cells were incubated for 2 h with rabbit anti-p-STAT-3 and anti-p-STAT-5 (Cell Signaling Technology). The cells were incubated for 40 min with the appropriate rabbit anti-IgG-Alexa Fluor 488-conjugated secondary antibodies (Invitrogen). The nuclei were stained with 20 μg/ml 4-6-diamidino-2-phenylindole (DAPI; Sigma) for 20 min. Light microscopy analyses were performed with a confocal laser scanning microscope (LSM 780 META, Zeiss, Germany).

CLONOGENIC PROGENITOR ASSAYS

Colony-forming units (CFUs) assays were performed by plating 5×10^4 murine total bone marrow cells with M-CSF (10 ng/ml) or G-CSF (10 ng/ml) in methylcellulose-based medium (Methocult M3134; Stem Cell Technologies) with signaling inhibitors of PKC (GF109023), CaMKII (KN62), PI3K (Wortmanin), MEK (PD98059), PLC (U73122) and nuclear factor of activated T-cells (NFAT) (VIVIT). The cells were plated in 35 mm diameter dishes under 5% CO_2 at 37°C. The colonies were quantified after 7 days of culture using a dark-field microscope.

GENE EXPRESSION ANALYSIS

Quantitative real-time PCR (qRT-PCR) was used to determine expression levels of distinct transcription factors. Briefly, cells were lysed in presence of β-mercaptoethanol and total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Venlo, Netherland) and then stored at −80°C until use. The RNA integrity was assessed by electrophoresis on agarose gels. cDNA was synthesized from 1 μg of total RNA with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) using random hexamer nucleotides. Standard curves for the primers were made to determine the amplification efficiencies of target and reference genes. Quantitative PCR was performed using the TaqMan system on an ABI Prism 7500 sequence detection system with 5 ng of cDNA. Target mRNA expression was normalized to beta-actin expression and expressed as a relative value using the comparative threshold cycle (Ct) method ($2^{\Delta\Delta Ct}$) according to the manufacturer's instructions. Expression levels from genes of interest were normalized to unstimulated cells and presented as fold change. The primers used were: Ikzf3 (Mm01306723_m1), GATA-1 (Mm01352636_m1), GATA-2 (Mm00492301_m1), GATA-3 (Mm00484683_m1), Sfpi-1/PU.1 (Mm00488142_m1), C/EBPα (Mm00514283_s1), and NF-E2 (Mm00801891_m1). All of them were purchased from Life Techologies (Van Allen Way, CA).

GENE SILENCING BY SMALL INTERFERENCE RNA (siRNA)

Mononuclear cells were isolated using Ficoll Histopaque (1.077 g/cm^3). The cells were then subjected to lineage depletion using immunomagnetic beads and a mixture of lineage antibodies (anti-B220, anti-Ter119, anti-Gr-1, anti-Mac-1, anti-CD3, MiltenyiBiotec, San Diego, CA). The Lin^- cells were labeled with anti-c-Kit (Miltenyi Biotec); then, $Lin^-c\text{-}Kit^+$ cells, which contain hematopoietic stem/progenitor cells, were isolated in a magnetic column. $Lin^-c\text{-}Kit^+$ cells (2×10^4 cells/well) were plated into 6-well plates for 18 h in IMDM, 2% FBS and siRNA for PLCγ1 or PLCγ 2 (both Santa Cruz Biotechnology, USA). siRNA reagents were transfected using the manufacturer's recommended protocol at a final concentration of 1,250 ng/X cells. The cultures were maintained at 37°C under 5% CO_2. The cells were collected, fixed with 2% paraformaldehyde for 30 min, washed with 0.1 M glycine, permeabilized with 0.01% saponin for 15 min and washed with PBS. The cells were incubated for 2 h with rabbit anti-PLCγ1 or anti-PLCγ2 (Cell Signaling Technology). The cells were incubated for 40 min with the appropriate rabbit anti-IgG-Alexa Fluor 488-conjugated secondary antibodies (Invitrogen). These data analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson). For CFU assays, 2×10^3 cells were collected per well and plated into 35 mm dishes with M-CSF (10 ng/ml) or G-CSF (10 ng/ml) in methylcellulose-based medium. The colonies were quantified after 7 days of culture using a dark-field microscope.

STATISTICAL ANALYSIS

Data are expressed as the mean ± standard error of the mean (SEM). Statistical comparisons were performed by using Student's *t*-test or analysis of variance (ANOVA). Values of $P < 0.05$ were considered statistically significant. All graphic data represent at least three independent experiments.

RESULTS

$Ca^{2+}{}_i$ SIGNALING INHIBITORS REDUCE CFU INDUCED BY M-CSF AND G-CSF

We have clarified the physiological relevancy of the $Ca^{2+}{}_i$ signal transduction pathway elicited by M-CSF and G-CSF. These are two well-known cytokines that simultaneously induce proliferation and monocytic or granulocytic differentiation, respectively [Bourette and Rohrschneider, 2000; Jack et al., 2009] (Table I; Fig. 1A), without reducing the hematopoietic stem/progenitor population in long-term cultures ($Gr\text{-}1^-Mac\text{-}1^-c\text{-}Kit^+$) (Fig 1B). The concentration of cytokines used in this study was previously tested in other reports [Paredes-Gamero et al., 2008; Jack et al., 2009; Barbosa et al., 2011; Leon et al., 2011]. In addition, the expression of known transcription factor associated with myeloid/lymphoid differentiation was evaluated in $c\text{-}Kit^+$ population from LTBMC after stimulation for 30 min with cytokines (Fig. 1C). M-CSF and G-CSF increase the expression of C/EBPα, but not alter expression of transcription factor associated to lymphoiesis (Ikaros, GATA2 and GATA3) nor erythropoiesis (GATA1). In addition, a reduction of Secreted frizzled-related protein 4 (SFRP4) a transcription factor associated with Wnt signaling that regulate expansion and maintenance of stem was observed [Reya et al., 2003]. Expression of PU.1 and NF-E2, transcription factor associated to myeloipoiesis was not statistically increased (Fig. 1C).

To identify participation of Ca^{2+} signaling, we used pharmacological inhibitors and verified the growth of CFU in methylcellulose that was induced by M-CSF or G-CSF. The inhibitors U73122 (5 μM), GF109203 (10 nM), and KN-62 (1 μM) were used to block the

TABLE I. M-CSF and G-CSF Promote Myeloid Differentiation in LTBMCs

	Macrophages	Monocytoids	Neutrophils	Immature forms	Blasts
Control	28 ± 3	29 ± 4	22 ± 2	18 ± 1	3 ± 1
G-CSF	35 ± 2	14 ± 3*	28 ± 4	17 ± 3	6 ± 1*
M-CSF	52 ± 4*	21 ± 3	9 ± 2*	14 ± 2	4 ± 1

LTBMCs were stimulated with M-CSF (10 ng/ml) or G-CSF (10 ng/ml) for 3 days. The evaluation of populations present was performed in cells stained with Giemsa/May-Grünwald.

These data are expressed as the mean ± SEM, $^*P < 0.05$. *The statistical analysis was performed against a control. ANOVA test; n = 5.

activation of PLC, PKC, and CaMKII, respectively. Our results demonstrated that the inhibition of PLC and PKC promoted a significant reduction in CFU number (~45%) in comparison with control cultures with control cultures that were treated with both cytokines (Fig. 2A and B). However, the inhibition of CaMKII only reduced the CFU formed by G-CSF (Fig. 2B). Moreover, other important signaling pathways in hematopoiesis such as MEK/ERK and PI3K were also blocked. The use of the MEK inhibitor, PD98059, exclusively reduced the percentages of CFU induced by M-CSF, but the effect of G-CSF was not altered (Fig. 2A and B). Wortmannin, a PI3K inhibitor, did not affect the number of colonies formed by M-CSF or G-CSF (Fig. 2A and B).

M-CSF AND G-CSF ACTIVATE INTRACELLULAR Ca^{2+}-DEPENDENT KINASES

To corroborate the pharmacological assay, specific antibodies against active forms (phosphorylated proteins) were used. LTBMCs were stimulated by M-CSF or G-CSF at 37°C for 5 min, and the phosphorylation of proteins was quantified by flow cytometry. We have previously shown a rapid and transient activation of intracellular pathways by cytokines in the hematopoietic stem/progenitor population [Paredes-Gamero et al., 2008; Jack et al., 2009; Leon et al., 2011]. We observed that the PLCγ1 isoform was not activated by M-CSF or G-CSF, whereas the phosphorylation of the PLCγ2 isoform was detected after stimulation with either M-CSF or

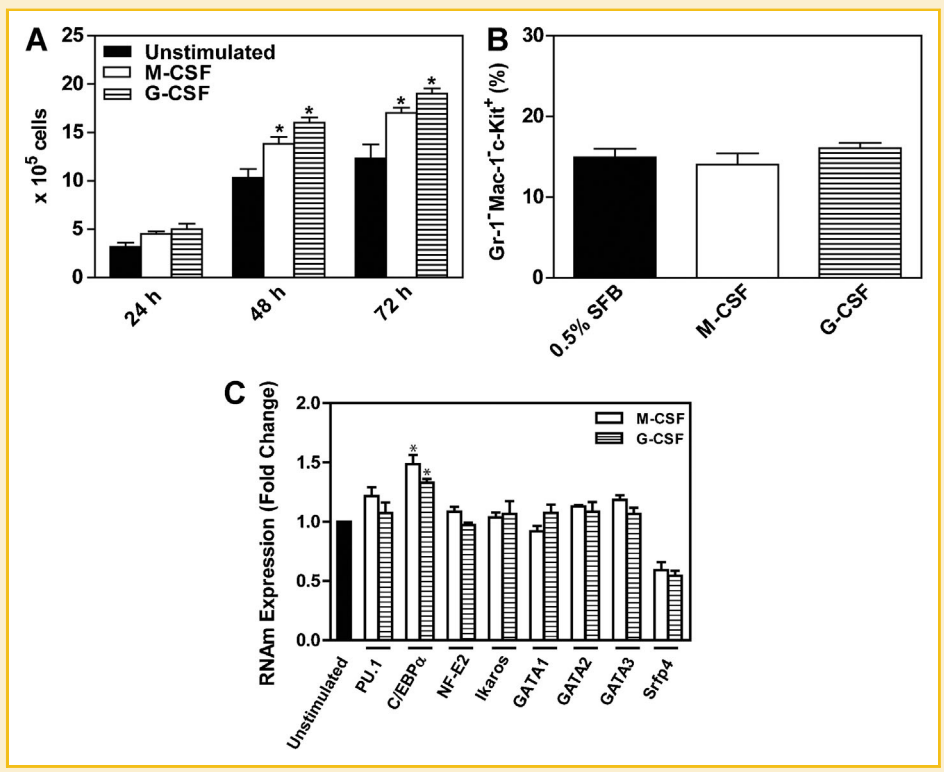

Fig. 1. M-CSF and G-CSF induce proliferation without decreasing the hematopoietic stem/progenitor population. LTBMCs were cultivated for 24 h in IMDM medium with 0.5% FBS and then stimulated with M-CSF (10 ng/ml) or G-CSF (10 ng/ml). A: Total cell counts were performed at 24, 48, and 72 h in a Neubauer chamber using trypan blue for cell death exclusion. B: The percentages of hematopoietic stem/progenitor population were assessed by flow cytometry 3 days after stimulation with both cytokines. M-CSF and G-CSF did not alter the percentage of the hematopoietic stem/progenitor population. C: Expression levels from transcription factors were performed by qRT-PCR. These data are expressed as the mean ± SEM, $^*P < 0.05$, ANOVA test, n = 5.

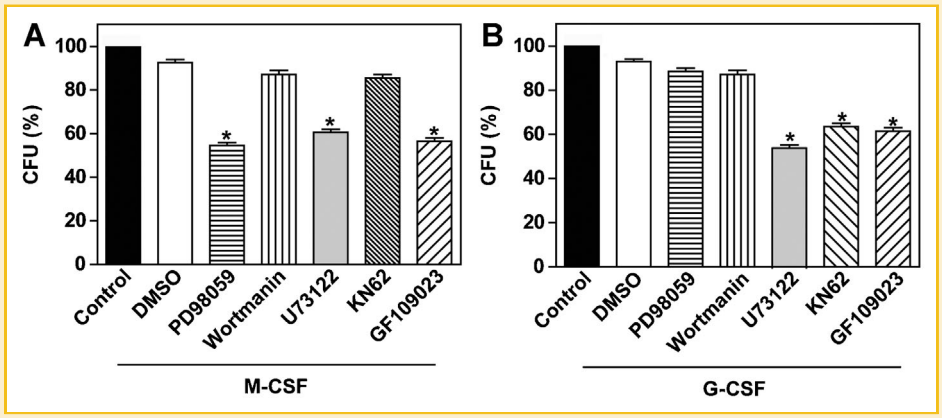

Fig. 2. Ca^{2+} signaling pathway inhibitors promote the decrease of CFU. Murine hematopoietic cells were incubated using methylcellulose medium with (A) M-CSF (10 ng/ml) or (B) G-CSF (10 ng/ml) plus MEK (PD98059), PI3K (Wortmanin), PLC (U73122), CaMKII (KN-62), or PKC (GF109023) inhibitors at 37°C in a humidified atmosphere with 5% CO_2. The colonies were counted after 7 days of culture. These data are expressed as the mean ± SEM, $^*P < 0.05$, n = 4.

G-CSF. The activation of PKC was also observed after stimulation with either cytokine. However, CaMKII was activated exclusively by G-CSF (Fig. 3A), corroborating the effect observed with the inhibitor KN-62.

To confirm the participation of PLCγ2, hematopoietic stem/progenitor cells were transfected with siRNA that targeted PLCγ1 or PLCγ2, and their functional roles were confirmed by CFU assays. Hematopoietic stem/progenitor cells were transfected with siRNA, and the expression of total PLCγ1 or PLCγ was quantified by flow cytometry after 18 h (Fig. 3B). The siRNA were able to partially reduce the expression of both PLCγ, and PLCγ2. Next, we tested the functional clonogenic capacity of hematopoietic stem/progenitor cells downregulated by PLCγ1 and PLCγ2. Only cells with down-regulated PLCγ2 had their clonogenic capability reduced by 50% in response to either cytokine; PLCγ1 siRNA did not affect the number of CFUs formed (Fig. 3C). To verify if the reduction observed occurs by cell death, the viability of colonies was assessed using Annexin-V and Propidium Iodide in an Image Cytometer (Tali, Invitrogen). Viability around 91% was observed in all groups (data not shown).

Additionally, the participation of PI3K, p38, and Jun N-terminal kinase was evaluated. The stimulation of LTBMCs with M-CSF and G-CSF did not promote the activation of p38, JNK, or AKT. However, the phosphorylation of ERK1/2 was observed in response to M-CSF, but not to G-CSF (Fig. 4).

G-CSF ACTIVATES Jak-1/STAT-3 AND PU.1, AND M-CSF ACTIVATES NFAT

Several transcription factors contribute to myeloid lineage specification. Therefore, we investigated the participation of some transcription factors associated with hematopoiesis and Ca^{2+} signaling. Figure 5A shows that G-CSF increases the expression of PU.1, but PU.1 expression was increased after 30 min of stimulation with M-CSF. c-Jun, a transcription factor activated by JNK, was not activated by either cytokine (Fig. 5A). Because the G-CSF receptor is a cytokine receptor without intrinsic kinase activity, we investigated the activation of the Jak/STAT pathway. G-CSF was able to activate Jak-1 and STAT-3, but not Jak-2 and STAT-5 (Fig. 5B). VIVIT (1 μM), a selective inhibitor of calcineurin-NFAT signaling, was also tested; M-CSF-induced CFUs were only reduced by 40% when NFAT was inhibited (Fig. 5C). Additionally, the translocation of NFAT and STATs to the nucleus was monitored in LTBMC cultures by confocal microscopy. LTBMCs were stimulated with G-CSF or M-CSF for 10 min; unstimulated and stimulated samples are shown. A higher expression NFAT1 was observed, even in unstimulated sample (Fig. 5D). On other hand, an increase of expression of NFAT2 was observed after M-CSF stimulation, but was not possible see clearly a translocation of NFAT to the nucleus (Fig. 5E). In Figure 5F, the top image corresponds to a central region from long-term cultures, and bottom image corresponds to a lateral area that can be preferentially observed in stromal cells. An increase of the activated form of STAT-3 and its translocation into the nucleus was observed, but STAT-5 was not activated (Fig. 5F and G).

To connect the Ca^{2+} signaling elicited by G-CSF and the Jak-1/STAT-3 pathway, we utilized U73122, a PLC inhibitor. U73122 was able to reduce STAT-3 phosphorylation and block the increase of PU.1 levels (Fig. 6A). However, these effects were not confirmed using GF109203, a PKC inhibitor (Fig. 6B).

DISCUSSION

Myeloid differentiation is regulated by a complex interaction of multiple cytokines. It has been reported that following the engagement of a cytokine to its cognate receptor, a number of intracellular signal transduction proteins become activated, including the Ca^{2+}-dependent kinases [Cullen and Lockyer, 2002; Irish et al., 2004; Krutzik et al., 2005; Paredes-Gamero et al., 2012]. Although multiple cytokines regulate the proliferation and survival of HSCs and progenitor cells, few reports have explored the role of these cytokines in the myeloid lineage-specific commitment events. Jack and collaborators directly compared the signal transduction of M-CSF and G-CSF receptors using the Ba/F3 lineage, an immature

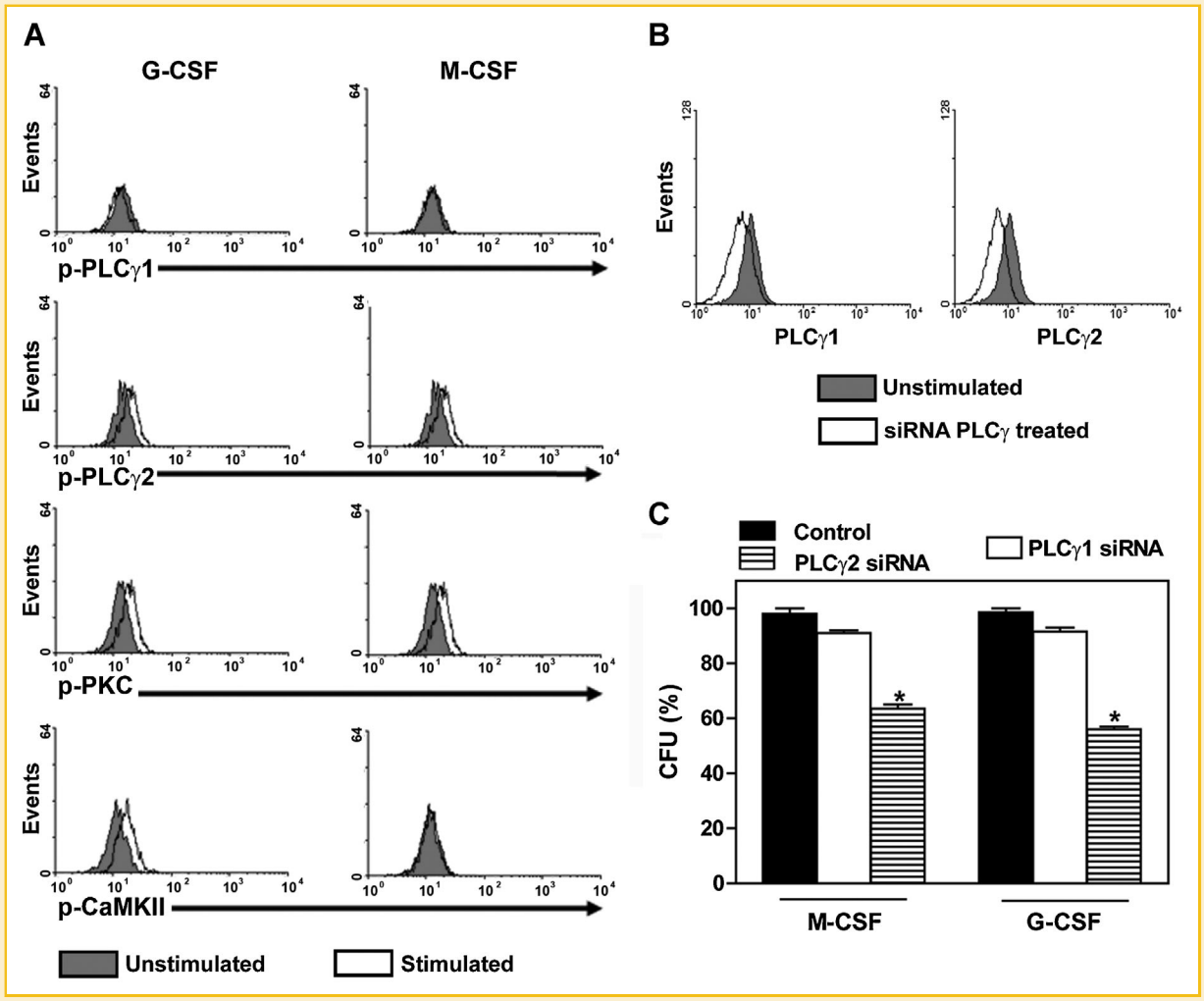

Fig. 3. The role of Ca^{2+} signaling in mediating the effects of M-CSF and G-CSF. LTBMCs were incubated for 24 h in IMDM medium with 0.5% FBS and then stimulated with M-CSF (10 ng/ml) or G-CSF (10 ng/ml) for 5 min. A: PKC and PLCγ2 were phosphorylated in response to both cytokines, while PLCγ1 was not activated. CaMKII was activated exclusively by G-CSF. The activation of proteins was evaluated in a hematopoietic stem/progenitor population by flow cytometry. The filled and open histograms represent unstimulated or stimulated samples, respectively. These data are representative of at least three experiments. B: $Lin^{-}c\text{-}Kit^{+}$ cells were transfected with PLCγ1 or PLCγ2 siRNA for 18 h. The quantification of PLCγ1 and PLCγ2 expression after siRNA transfection was performed by flow cytometry. C: The cells were collected, stimulated with M-CSF (10 ng/ml) or G-CSF (10 ng/ml), and plated in methylcellulose medium for the CFU assay. The downregulation of PLCγ2 reduces the number of CFUs stimulated by M-CSF and G-CSF. The colonies were counted after 7 days of culture. These data are expressed as the mean ± SEM, $^{*}P < 0.05$, n = 3.

myeloid cell line, and showed that M-CSF activates ERK, thereby inducing higher levels of c-Fos and C/EBP, whereas G-CSF stimulates SHP2 phosphorylation and STAT-3 [Jack et al., 2009]. However, the differentiation of HSCs results in a signaling switch and crosstalk between these pathways that might vary with cell-stage specificity in a temporal manner and promote an alteration of their physiological role [Wandzioch et al., 2004].

Ca^{2+} is a ubiquitous cellular signaling molecule important in several processes. An early study showed that different cytokines related with myeloid and lymphoid lineages, such as IL-3, GM-CSF, M-CSF, G-CSF, SCF, and IL-7, promote oscillatory Ca^{2+} signals that lead to the proliferation of hematopoietic stem/progenitor cells [Paredes-Gamero et al., 2008]. In addition, a subsequent study showed the importance of Ca^{2+} signaling in myeloid differentiation [Leon et al., 2011]. These studies have associated Ca^{2+} signaling elicited by IL-3 and GM-CSF with the activation of kinases that are Ca^{2+}-dependent, and more importantly, those studies demonstrated the crosstalk between Ca^{2+} signaling and the MEK/ERK1/2 pathway in murine and human hematopoietic stem/progenitor cells [Paredes-Gamero et al., 2008; Leon et al., 2011]. Among the Ca^{2+} signaling proteins investigated in these reports, PLCγ2 has been described as an important molecule activated by IL-3 and GM-CSF and is associated with myeloid proliferation and the differentiation of hematopoietic stem/progenitor cells [Leon et al., 2011]. Similarly, M-CSF and G-CSF promoted the activation of PLCγ2, indicating its importance in myeloid differentiation (Fig. 3).

This report demonstrated that the effects of M-CSF and G-CSF are mediated by common upstream $Ca^{2+}{}_{i}$ signaling, although some

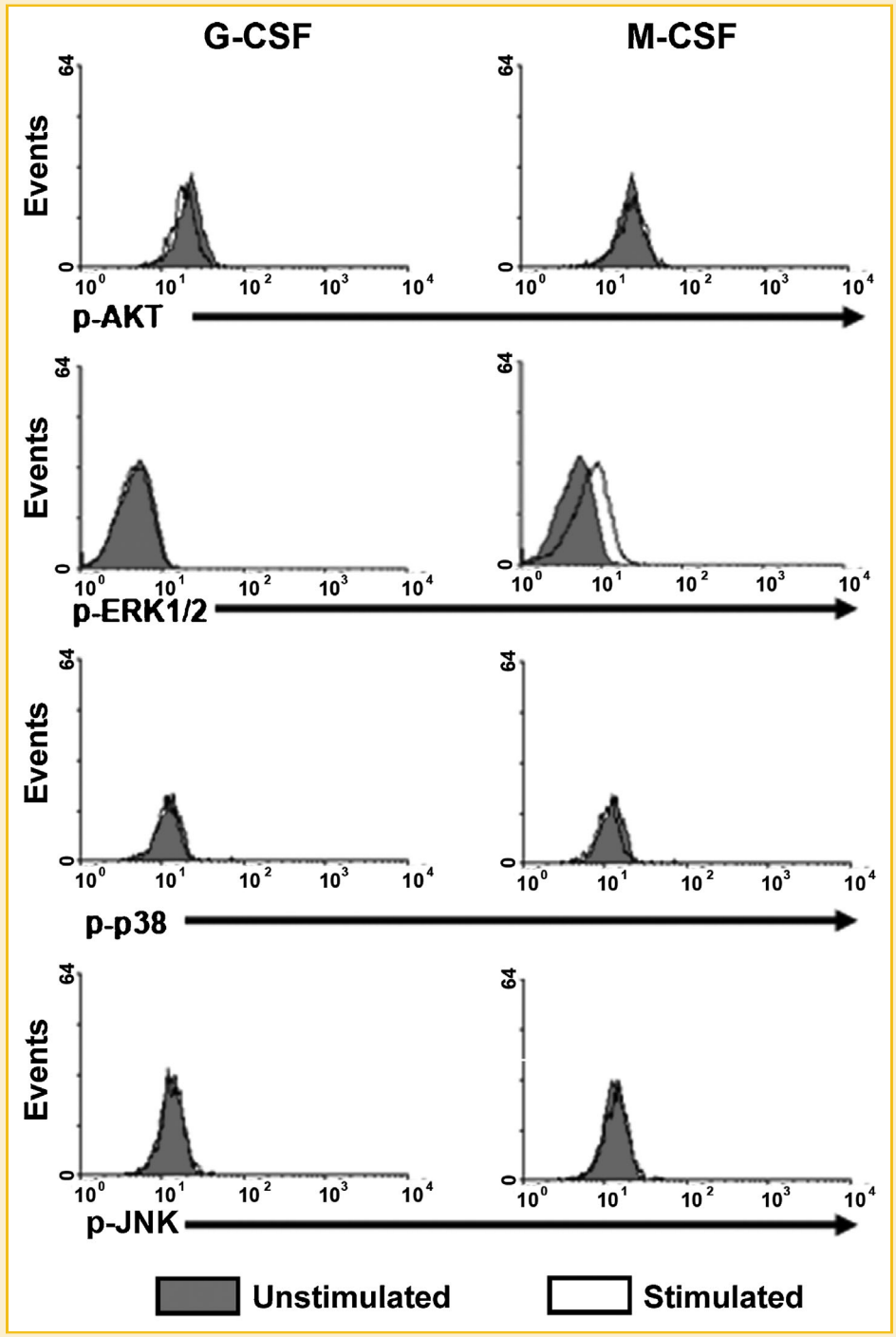

Fig. 4. ERK1/2 is activated by M-CSF, but not by G-CSF, in hematopoietic stem/progenitor cells. LTBMCs were incubated for 24 h in IMDM medium with 0.5% FBS and then stimulated with M-CSF (10 ng/ml) or G-CSF (10 ng/ml) for 10 min. The phosphorylation of proteins in the hematopoietic stem/progenitor population was assessed by flow cytometry. The p-38, JNK, and AKT pathways were not activated, but ERK1/2 phosphorylation in response to M-CSF was observed. The filled and open histograms represent unstimulated and stimulated samples, respectively. These data are representative of at least three experiments.

important differences in their downstream signals were observed. A possible common upstream pathway for M-CSF and G-CSF was elucidated because both are sensitive to PLC and PKC inhibitors (Fig. 2) and both activate PLCγ2 and PKC (Fig. 3). Similar results were previously observed in hematopoietic stem/progenitor cells stimulated with IL-3 and GM-CSF, two myeloid cytokines [Leon et al., 2011], and in Ba/F3 cells [Jack et al., 2009]. Therefore, these results suggest that both PLCγ2 and PKC are important to granulocytic/monocytic proliferation and differentiation.

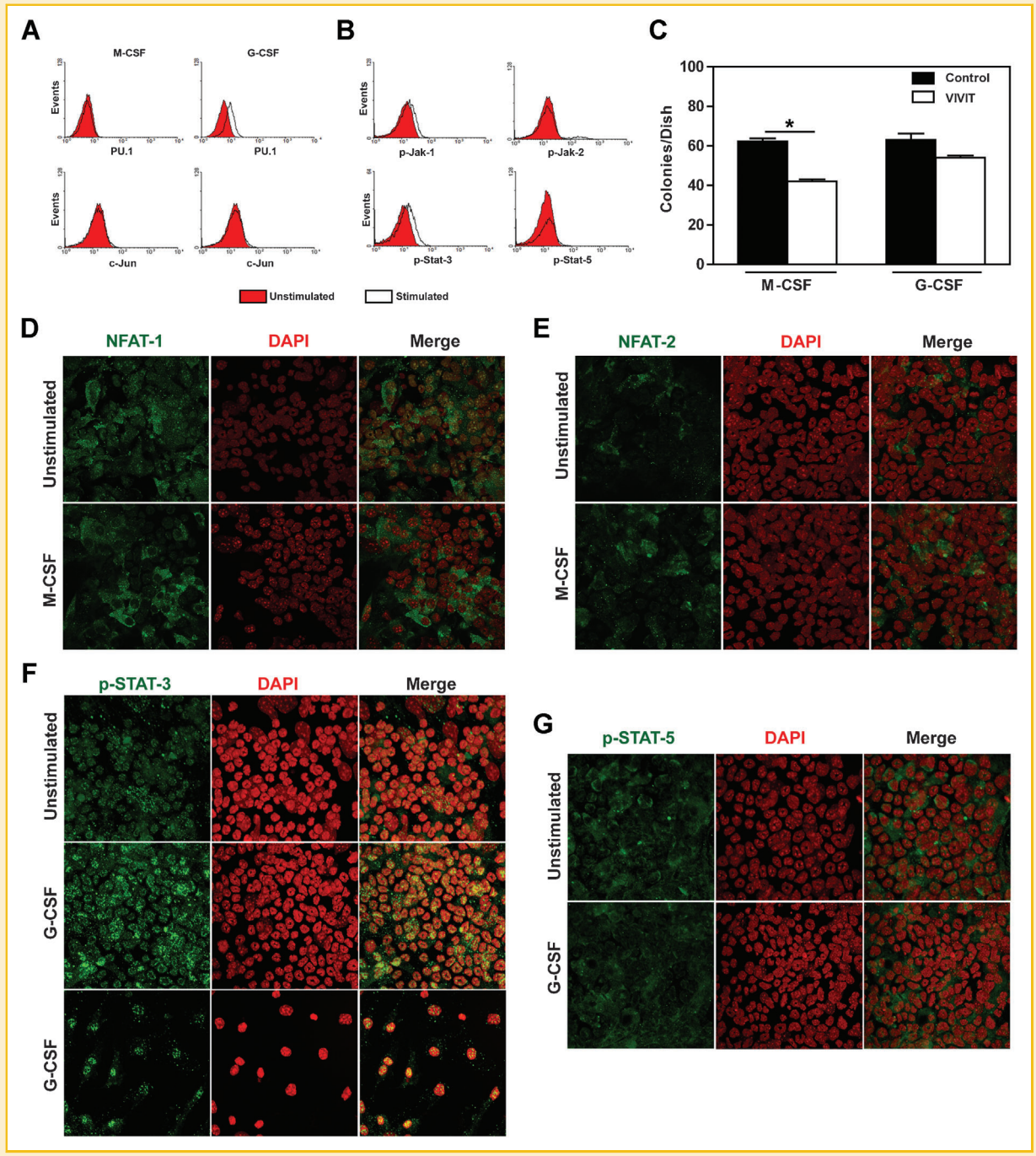

Fig. 5. STAT-3 and PU.1 transcription factors are related to granulopoiesis, while NFAT is required for monocytic commitment. LTBMCs were incubated for 24 h in IMDM medium with 0.5% FBS and then stimulated with M-CSF (10 ng/ml) or G-CSF (10 ng/ml) for 30 min. A and B: The samples were evaluated by flow cytometry in a hematopoietic stem/progenitor population. A: The expression of c-Jun was not altered by either cytokine, whereas PU.1 expression was preferentially increased by G-CSF. B: G-CSF activates Jak-1, but not Jak-2. The filled and open histograms represent unstimulated or stimulated samples, respectively. C: VIVIT peptide, an NFAT transcription factor inhibitor, reduced the number of CFU in the methylcellulose assay. D–G: The expression and localization of proteins were evaluated by confocal microscopy in LTBMCs after stimulation. D: NFAT-1. E: NFAT-2, an increase of expression of NFAT-2 can be observed. G-CSF induced the translocation (F) of STAT-3 into the nucleus, but not (G) STAT-5. F: The top image is an unstimulated sample; the middle image is a stimulated sample in the central region of the LTBMCs; and the bottom image is a stimulated sample in an section where the stromal cells can be observed. The images were captured by a confocal microscope (LSM 780 META). Scale bar, 20 μm. The secondary antibody anti-IgG-Alexa Fluor 488 (green) was used for the visualization of active proteins. The nuclei were stained with DAPI (red). These data are representative of at least three experiments.

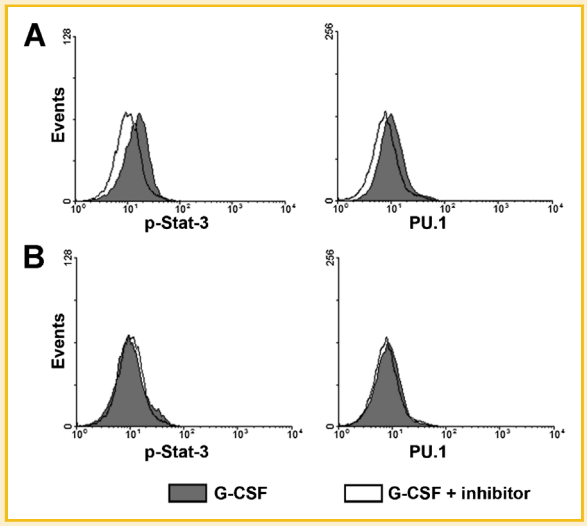

Fig. 6. The PLC inhibitor, U73122, reduces the phosphorylation of STAT-3. A: Pretreatment with U73122 leads to a decrease in the levels of STAT-3 and PU.1 in hematopoietic stem/progenitor cells. B: This result was not found using the PKC inhibitor, GF109203. The filled and open histograms represent stimulated samples incubated (filled) or not incubated (open) with the inhibitor. These data are representative of at least three experiments.

Among the differences observed in the signaling triggered by M-CSF were ERK1/2 activation and the participation of NFAT. In the hematopoietic system, ERK1/2 plays an essential role in controlling the proliferation and differentiation of hematopoietic cells and myeloid progenitors [Hsu et al., 2007; Bourgin-Hierle et al., 2008; Geest and Coffer, 2009; Geest et al., 2009; Jack et al., 2009; Leon et al., 2011; Nogueira-Pedro et al., 2011]. The ERK pathway is important for the proliferation and maturation of distinct hematopoietic lineages [Pages et al., 1999; Hsu et al., 2007; Leon et al., 2011; Nogueira-Pedro et al., 2011]. Our data have shown MEK/ERK1/2 participation in hematopoietic stem/progenitor cells, which is consistent with previous reports [Hsu et al., 2007; Jack et al., 2009]. Additionally, it is well established that growth factors can induce Ca^{2+} signals, thus activating gene transcription via the NFAT family of proteins, to regulate cell cycle progression and tumorigenic invasion [Yiu and Toker, 2006; Buchholz and Ellenrieder, 2007]. In this report, we also showed that NFAT participates in the regulation of macrophage/monocyte commitment (Fig. 5C), and NFAT-2 is upregulated after stimulation with M-CSF (Fig. 5E). However, neither CaMKII nor NFAT appear to participate in the effects induced by G-CSF.

In some aspects, the intracellular pathways triggered by G-CSF diverge from the mechanisms activated by M-CSF. The G-CSF receptor is a cytokine receptor without intrinsic tyrosine kinase activity that requires Jak to phosphorylate its tyrosine residues. It has

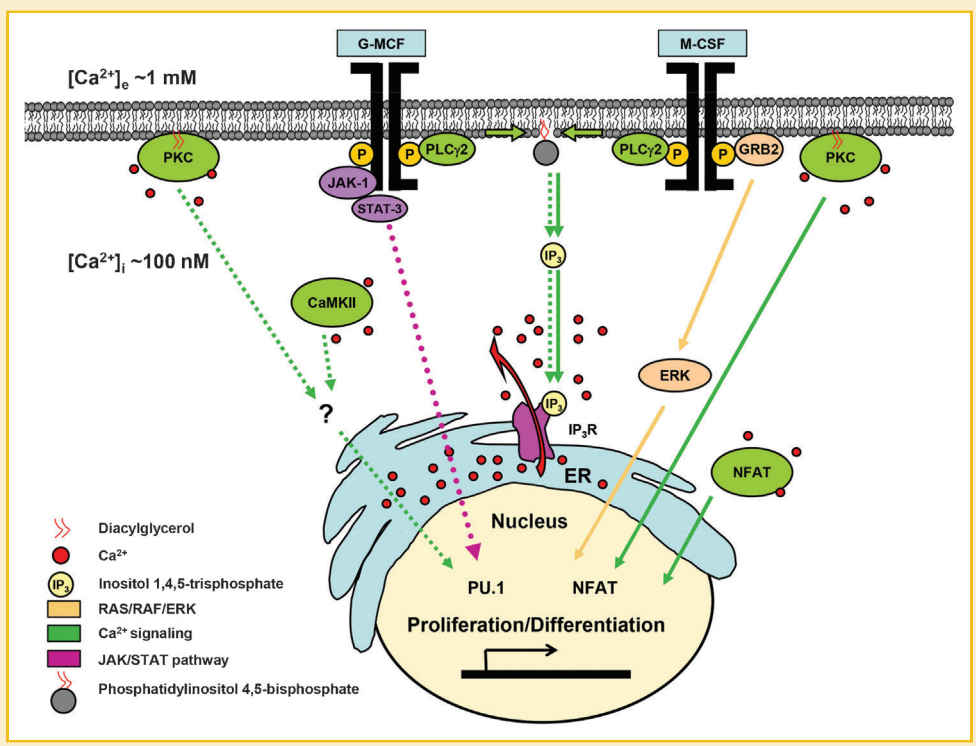

Fig. 7. Proposed model of the signaling mechanism triggered by G-CSF and M-CSF in hematopoietic stem/progenitor cells. Dotted line represents signaling triggered by G-CSF. Solid line represents signaling triggered by M-CSF. The binding of the G-CSF to its receptor triggers activation of JAK1 that phosphorylate tyrosine residues on the receptor. The residues phosphorylated are recognized by proteins such as STAT-3 and PLCγ2. Activation of PLCγ2 produces IP_3 by the cleavage of phosphatidylinositol 4,5-bisphosphate. IP_3 binds to its receptor (IP_3R) releasing intracellular Ca^{2+} from endoplasmic reticulum (ER). Intracellular Ca^{2+} binds actives PKC and CaMKII. The signaling triggered by G-CSF promotes the increase of transcription factor PU.1. All these pathways modulate granulocytic differentiation. In addition, M-CSF induces monocytic differentiation by activation of PLCγ and ERK pathways. Ca^{2+} released by IP_3 also actives PKC, but not CaMKII. The transcription factor NFAT is also activated in monocytic differentiation. Thus, some proteins are activated in granulocytic and monocytic differentiation such as PLCγ2 and PKC and other seem to be specific to granulocytic and monocytic lineage commitment.

been shown that G-CSF activates the Jak-1 subtype and simultaneously activates PLCγ2 and PKC, similar to M-CSF. Additionally, CaMKII, a Ca^{2+}/CaM-dependent kinase, was also activated by G-CSF (Figs. 2 and 3), as well as by GM-CSF in hematopoietic stem/progenitor cells [Paredes-Gamero et al., 2008; Leon et al., 2011]. We demonstrated that PLCγ2 is important for the activation of the transcription factors STAT-3 and PU.1 because PLCγ2 inhibition causes a reduction of STAT-3 activation and blocks the increased expression of PU.1 (Fig. 6).

To summarize, we corroborated the importance of PLCγ2 and PKC in monocyte/granulocyte commitment and elucidated differences in the proliferation and differentiation that are caused by the signaling cascades generated by G-CSF and M-CSF. We demonstrated that the participation of ERK1/2 and NFAT is associated with the effects elicited by M-CSF and that the participation of JAK1, STAT-3 and PU.1 is associated with the effects elicited by G-CSF. A schematic model of the mechanism involved G-CSF and M-CSF is showed in Figure 7. This study helps to clarify the link between the $Ca^{2+}{}_i$ signal transduction pathway and its biological functions in monocytic and granulocytic lineage commitment.

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