
The Critical Role of the Colony-Stimulating Factor-1 Receptor in the Differentiation of Myeloblastic Leukemia Cells

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Abstract

How diverse stimuli control hemopoietic lineage development is unknown. An early event during induction of macrophage differentiation in the myeloblastic leukemia M1 cell line by different stimuli, such as leukemia inhibitory factor (LIF) and interleukin-6 (IL-6), is expression of the colony-stimulating factor-1 receptor (CSF-1R). We report that expression of active CSF-1R in M1 cells accelerated their subsequent terminal differentiation into macrophages in response to LIF and IL-6 when compared with cells lacking the CSF-1R or expressing the receptor with compromised kinase activity; however, there was no requirement for signaling through the CSF-1R, for example, via endogenous CSF-1, during the actual LIF-induced and IL-6-induced differentiation stage. Differences were noted in the signaling pathways downstream of the LIF receptor depending on the presence of the CSF-1R. Both LIF and IL-6 gave an additive response with CSF-1, consistent with LIF and IL-6 acting via a different signaling pathway (signal transducer and activator of transcription 3 dependent) than CSF-1 (extracellular signal-regulated kinase dependent). Based at least on this cell model, we propose that terminal macrophage differentiation involves a critical priming or deterministic phase in which signaling by the CSF-1R prepares a precursor population for subsequent rapid terminal macrophage differentiation by diverse stimuli. We also propose that expression and activation of the CSF-1R explain much prior literature on macrophage lineage

commitment in M1 leukemic cells and may be important in controlling the progression of certain myeloid leukemias. (*Mol Cancer Res* 2008;6(3):458–67)

Introduction

Hemopoietic development along the various cell lineages occurs following proliferation and differentiation of precursor cells in an orchestrated way involving multiple growth factors and cytokines (1). The contribution of the instructive actions of cytokines, in addition to their generally viewed primary role in cell survival and proliferation, has not been well addressed (2, 3). Lineage commitment is believed to be due to expression of lineage-specific receptors at sufficient levels associated with parallel intracellular molecular changes. Under some conditions, paracrine and autocrine mechanisms seem to apply involving these lineage-specific receptors (4). Cross-talk at the level of growth factor/cytokine receptors has also been put forward as a mechanism for the interplay between different stimuli (5). One growth factor controlling macrophage lineage development in particular is macrophage colony-stimulating factor (M-CSF or CSF-1) acting through its proto-oncogene receptor tyrosine kinase, c-Fms (6, 7).

The murine myeloblastic leukemia M1 cell line is induced by several stimuli to undergo terminal macrophage differentiation, coupled to growth arrest and apoptosis of mature cells, and is a widely used model system in which to analyze the molecular mechanisms controlling hemopoietic cell differentiation and how blocks in these signals can result in disease onset. Two commonly used cytokine inducers of macrophage differentiation in the M1 cells are leukemia inhibitory factor (LIF) and interleukin-6 (IL-6; refs. 8, 9). Among several changes observed, one marker of macrophage differentiation appearing in the M1 cells in response to LIF (10, 11) and IL-6 (10, 12) is the CSF-1 receptor (CSF-1R). Using such prior information, we previously reasoned that transfection of CSF-1R into M1 cells would give rise to a cell population that would differentiate in response to added CSF-1 and provide a cell system to study CSF-1-induced differentiation, a major response *in vitro* of human bone marrow precursor cells and monocytes (13). We found that such transfection did lead to CSF-1-induced macrophage differentiation (14). What was interesting and perhaps surprising was the rapid response that could be observed even as soon as 24 to 48 h after CSF-1 addition. In other words, the starting M1 population seemed ready to differentiate into macrophages provided sufficient

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CSF-1R was present, suggesting that CSF-1R-mediated signaling might be a critical limiting component of the M1 cell differentiation program into macrophages.

Other approaches have resulted in the induction of a M1 macrophage differentiation program(s), which also includes the early appearance of CSF-1R. Two such examples are the constitutive expression of the transcription factors *ets-2* (15) and Wilms' tumor suppressor gene (16). Addition of LIF to both of these modified M1 populations resulted in rapid and dramatic macrophage differentiation when compared with that seen in the unmodified starting population. Based on these findings, it was concluded that there was another signal(s), in addition to those provided initially by LIF, for example, which was required for terminal macrophage differentiation (16).

Differentiation inducers in M1 cells usually give rise to macrophage-like cells, including even granulocyte-CSF (G-CSF; ref. 17), which might be expected to lead to granulocyte differentiation. Ectopic expression of the thrombopoietin receptor (c-Mpl; ref. 18) and the granulocyte macrophage-CSF (GM-CSF) receptor (19), followed by addition of the respective cognate ligands, also leads only to macrophage differentiation. These prior observations on CSF-1R induction during differentiation, susceptibility to rapid CSF-1-induced differentiation of M1 populations expressing CSF-1R, and macrophage-specific differentiation in response to a diverse range of agents suggested that CSF-1R induced in response to diverse stimuli might provide the required second signal for the terminal differentiation referred to above. A resulting prediction is that, like the M1 cells expressing *ets-2* and Wilms' tumor suppressor gene that have CSF-1R on their surface (15, 16), M1 cells engineered to express CSF-1R (14) might also rapidly differentiate into macrophage-like cells in response to LIF (and also perhaps to IL-6). Because CSF-1R⁺ cells have been reported in M1 populations (20), this concept might also explain the published "all or none" responsiveness of individual M1 clones to LIF (8, 9). We report here that LIF and IL-6 cause rapid induction of macrophage differentiation in such CSF-1R-expressing M1 cells when compared with a starting M1 population without this receptor. Evidence is presented for a model in which expression and activation of CSF-1R prepare the M1 cells for subsequent rapid macrophage differentiation in response to LIF (and IL-6); however, activation of the CSF-1R, for example, by endogenous CSF-1 does not seem to be required during the phase of rapid terminal differentiation induced by LIF.

Because CSF-1 is normally present in the circulation and is made by many tissues, macrophage lineage cells are likely to be continually exposed to it (6). The creation of CSF-1R-expressing M1 cells allows the effects of CSF-1 on macrophage differentiation by other inducers to be studied. We report that LIF and IL-6 potentiate the macrophage differentiation induced by CSF-1 even at optimal concentrations of the latter, thereby supporting a model involving cross-talk between separate signaling cascades downstream of activated CSF-1R.

Results

Rapid LIF-Induced and IL-6-Induced Macrophage Differentiation in M1 Cells Expressing CSF-1R

We engineered M1 cells to express the wild-type (WT) CSF-1R [previously termed M1/WT cells to distinguish them

from cells engineered to express mutant CSF-1R forms (14, 21, 22)] by depleting the low numbers of CSF-1R⁺ cells from a starting M1 cell population (10) followed by CSF-1R transfection (14). This prior depletion was necessary so that the influence of the transfected receptor could be ascertained more clearly. The CSF-1R-depleted population will be referred to here as the "parental" cells because they were used to derive the M1/WT cells that were subsequently used as pools of individual clones expressing similar CSF-1R levels (14).

LIF has been shown to induce a macrophage-like differentiation in M1 cells (8, 11). We compared the LIF-induced differentiation of M1/WT and the parental cells by several criteria. A macrophage-like morphology in the LIF-treated M1/WT cells appeared by day 2 (Fig. 1A) but was only slightly evident by day 4 in the LIF-treated M1 cells (Fig. 1B). A similar difference in the relative suppression of cell growth was

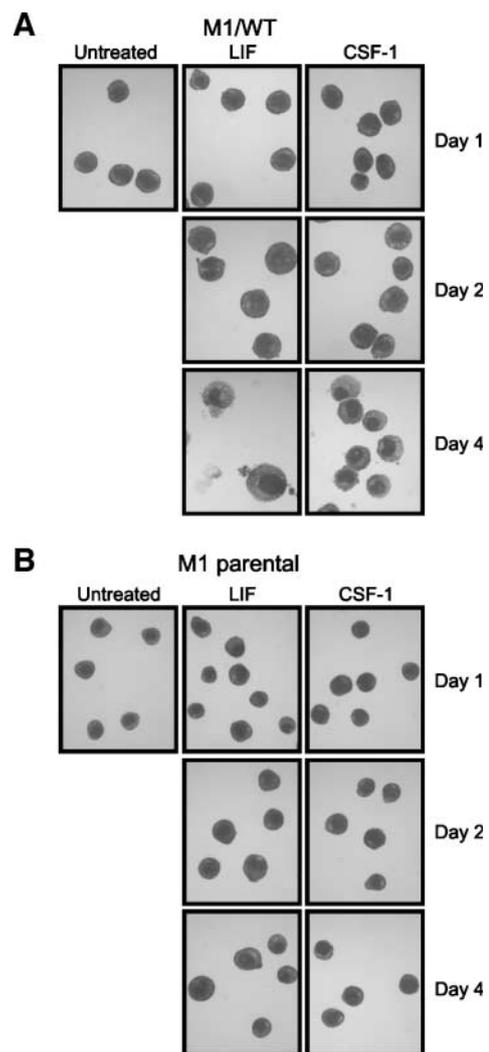


FIGURE 1. Rapid LIF-induced morphologic changes in M1 cells expressing CSF-1R. M1/WT (A) and M1 (B) parental cells (7×10^4 /mL) were cultured in the presence of LIF (10 ng/mL) or CSF-1 (5×10^3 units/mL) for the indicated number of days. Cytospins of the cells were then stained with Diff-Quik. The morphology changes from one experiment, which was repeated eight times, are presented.

also observed (Fig. 2A versus Fig. 2B); DNA synthesis measurements, measured by a 24-h pulse of [3 H]TdR at day 2 or 3, paralleled the cell number data and indicated changes in cell cycle status (data not shown). The data for the corresponding CSF-1–treated populations are provided (Figs. 1 and 2A and B) and confirm our previous findings (14). The effective dose of LIF for the M1/WT differentiation was in the range of 0.1 to 1 ng/mL. IL-6 gave similar results over the concentration range of 1 to 100 ng/mL (data not shown). Prior studies using colony assays as a readout for proliferation rate have indicated that both LIF and IL-6 can induce toxic effects on M1 cell populations, which are probably due to apoptosis associated with the development of the differentiated phenotype (8, 16, 17, 23). We observed that the viability of LIF-treated M1/WT cells was reduced even after a 2-day culture period and was reflected later in the growth rate suppression. When Annexin V/propidium iodide staining was used to monitor apoptosis by flow cytometry, it was found by this time that some apoptosis was beginning to occur following treatment with LIF (Fig. 2C) and IL-6 (data not shown) but not with CSF-1 (Fig. 2C). Apoptosis took significantly longer to be induced in the parental M1 cells (Fig. 2D; data not shown). When Mac-1 was used as a differentiation marker (22), its expression (percentage positive cells and median fluorescence intensity per cell) was first observed at day 1 in LIF-treated M1/WT cells but only by day 4 in the M1 parental counterparts (data not shown); however, quantification of Mac-1 expression in LIF-treated populations at later times (i.e., later than 2 days for M1/WT cells) was made difficult by the apoptosis occurring.

The Role of CSF-1R Signaling in LIF-Induced and IL-6–Induced Macrophage Differentiation

CSF-1R Mutation. We have previously shown that mutation of Tyr⁸⁰⁷ in the kinase domain of CSF-1R in CSF-1R–transfected parental M1 cells led to a dramatic loss in the extent of CSF-1–induced macrophage differentiation on account of compromised signal transduction (14, 21). To test further our model that expression and activation of CSF-1R prepare the M1 cells for subsequent rapid macrophage differentiation, we found that the M1 cells with the mutation in Tyr⁸⁰⁷ in the CSF-1R [M1/807 cells (21)] essentially failed to differentiate in response to LIF and IL-6 in the 2-day period examined, paralleling their response to CSF-1 (21); data for the effect of LIF and IL-6 on Mac-1 expression in M1/807 cells are shown in Fig. 3A as is the comparable data for the response to CSF-1.

Endogenous CSF-1. M1 cells can produce CSF-1 in culture medium (11, 12). An obvious possible mechanism for the preferential degree of differentiation of M1/WT over parental M1 cells in response to LIF and IL-6 is the contribution of this endogenous CSF-1 in a paracrine/autocrine manner. To test this possibility, a blocking monoclonal antibody (mAb) to the CSF-1R was added. In contrast to its suppressive effect on CSF-1–mediated differentiation in M1/WT cells, this anti-CSF-1R antibody failed to reduce the LIF-induced differentiation as monitored by reduction in the proliferation rate (Fig. 3B) and in the level of DNA synthesis (data not shown). In addition, the lack of effect of the anti-CSF-1R antibody on the LIF-induced changes in forward-side scatter profile can be seen in Fig. 3C.

If endogenous CSF-1 activity, whether constitutive or induced by LIF (11), was the mechanism for the action of LIF, then CSF-1R should be activated. At least over the time frame of 60 min, LIF (1 ng/mL), unlike CSF-1, did not enhance CSF-1R tyrosine phosphorylation, measured by Western blotting of cell lysates with anti-phosphotyrosine antibody, nor internalize cell surface CSF-1R, measured by flow cytometry, over the same time period (data not shown). These data suggest that the CSF-1R–dependent macrophage differentiation induced by LIF was not due to a paracrine/autocrine effect of secreted CSF-1; these data also suggest that LIF does not function by activating cell surface CSF-1R.

CSF-1R Kinase Activity. The data in Fig. 3A suggest that at some stage sufficient CSF-1R kinase activity is required for the

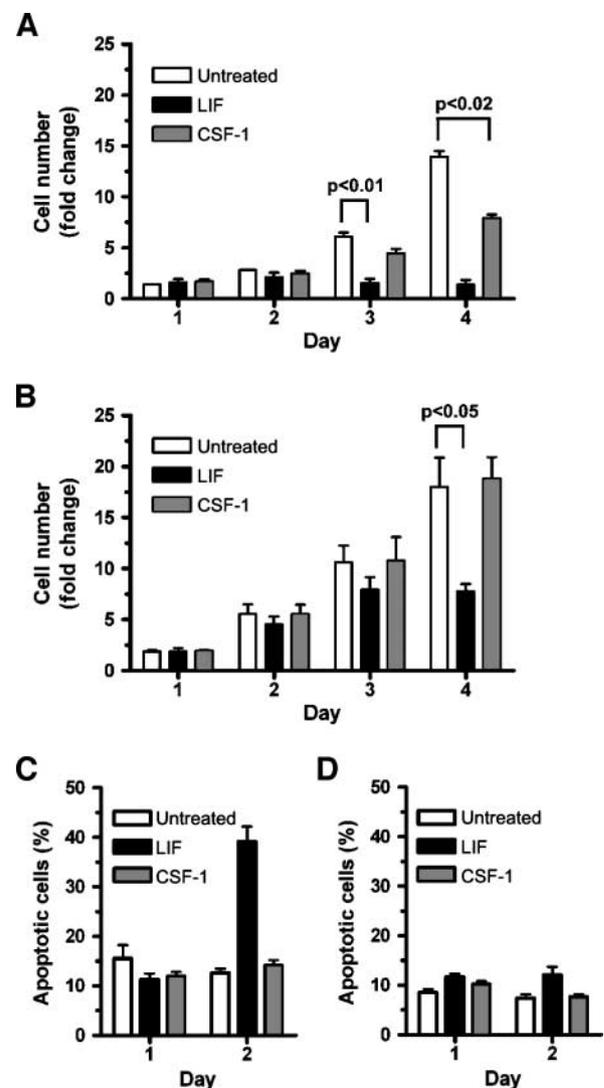


FIGURE 2. Rapid LIF-induced growth arrest and apoptosis in M1 cells expressing CSF-1R. M1/WT (**A** and **C**) and M1 parental (**B** and **D**) were cultured in the presence of LIF (10 ng/mL) or CSF-1 (5×10^3 units/mL) for the indicated number of days. **A** and **B**. Viable cells were counted and expressed as the fold change over the number of cells seeded at day 0. **C** and **D**. Percentage of apoptotic cells (propidium iodide⁺ and/or Annexin V⁺) after 1 and 2 d of culture was measured. Columns, mean of three independent experiments (triplicate cultures); bars, SE. *P* values were determined using the Student's *t* test.

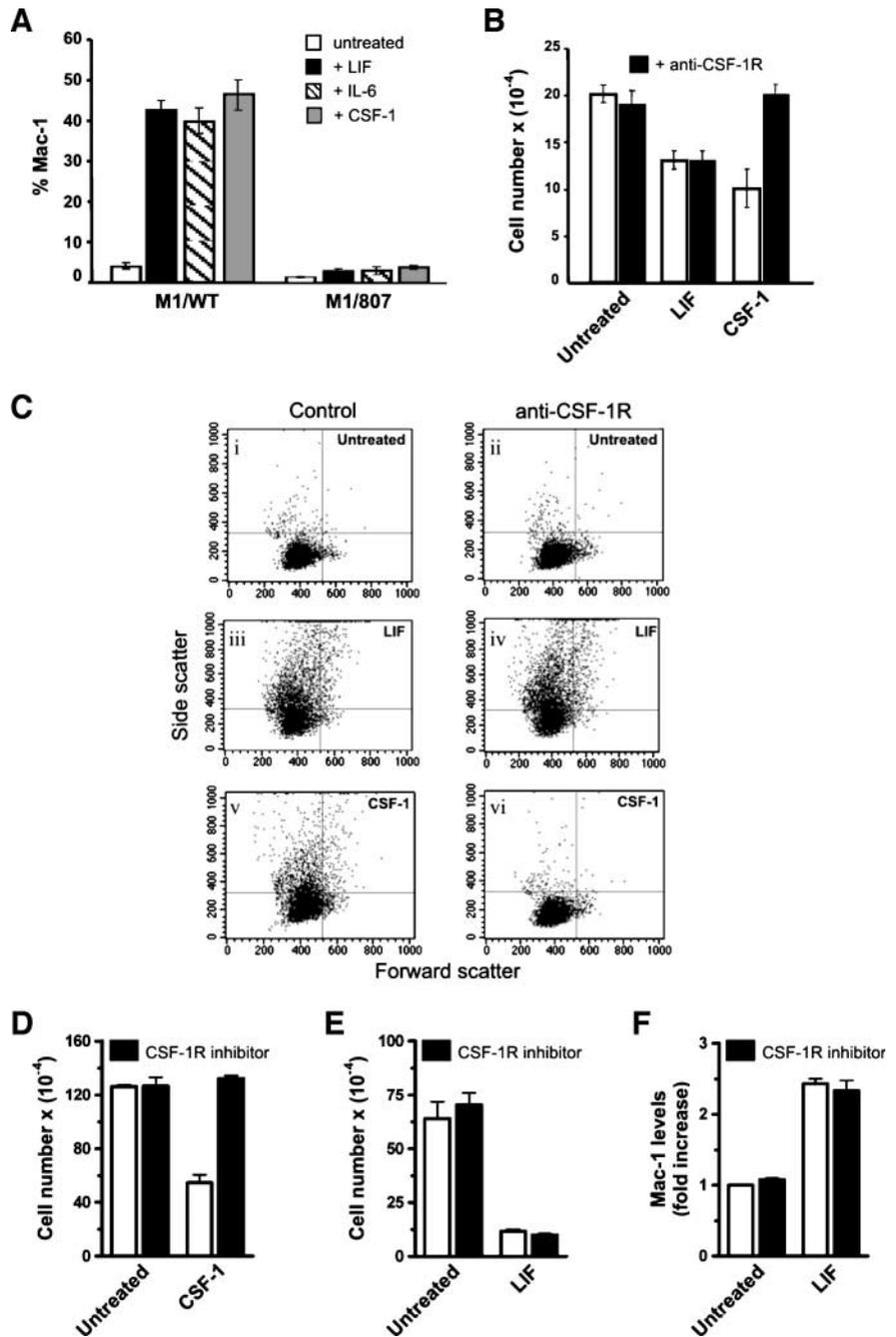


FIGURE 3. The role of CSF-1R signaling in LIF-induced and IL-6-induced differentiation in M1 cells expressing CSF-1R. **A.** M1/807 cells. M1/WT and M1/807 cells (2×10^5 /mL) were cultured for 2 d in the presence or absence of LIF (1 ng/mL), IL-6 (100 ng/mL), or CSF-1 (5×10^3 units/mL), and the percentage of Mac-1-positive cells was measured. Columns, mean of four independent experiments (triplicate cultures); bars, SE. **B and C.** Effect of anti-CSF-1R antibody blockade. M1/WT cells (5×10^3 /mL) were cultured in the presence or absence of LIF (1 ng/mL) or CSF-1 (5×10^3 units/mL) and in the presence or absence of anti-CSF-1R mAb. **B.** Viable cell numbers were enumerated after 4 d from triplicate cultures. Columns, mean of a representative experiment (triplicate cultures) that was repeated five times; bars, SE. **C.** M1/WT cells (2×10^5 /mL) were cultured in the presence or absence of LIF (1 ng/mL) or CSF-1 (5×10^3 units/mL) and in the presence or absence of anti-CSF-1R mAb. Forward and side scatter profiles after 2 d are presented. i, untreated cells, no mAb; ii, untreated cells + mAb; iii, LIF-treated cells, no mAb; iv, LIF-treated cells + mAb; v, CSF-1-treated cells, no mAb; vi, CSF-1-treated cells + mAb. This experiment was repeated six times. **D to F.** Effect of a CSF-1R kinase inhibitor. M1/WT cells (7×10^4 /mL) were untreated (white columns) or pretreated (black columns) with the CSF-1R kinase inhibitor (2.5 μ mol/L GW2580) for 60 min and then cultured for 3 d (**D** and **E**) or 2 d (**F**) in the presence or absence of CSF-1 (5×10^3 units/mL) or LIF (10 ng/mL). Viable cell numbers were enumerated (**D** and **E**), and Mac-1 levels were quantified by flow cytometry and expressed as the fold increase in mean fluorescence intensity over that at day 0 (**F**). Columns, mean of three independent experiments (triplicate cultures); bars, SE.

potentiation of the LIF-induced macrophage differentiation by CSF-1R expression. To explore the role of CSF-1R kinase activity further, we added a CSF-1R kinase inhibitor, GW2580, which has recently been used to attenuate CSF-1R signaling both *in vitro* and *in vivo* (24). GW2580 completely inhibited the CSF-1-stimulated differentiation in M1/WT cells (Fig. 3D); however, it failed to suppress the LIF-induced differentiation, as judged by cell growth (Fig. 3E) and Mac-1 expression (Fig. 3F). It is interesting that, given the poor response of the M1/807 cells to LIF (Fig. 3A), CSF-1R kinase activity in the M1/WT cells is not required during the phase of LIF-induced rapid differentiation (see Discussion).

LIF-Dependent Signal Transduction in M1 Cells Expressing CSF-1R

Paradoxically, the above data indicate that, although CSF-1R kinase activity did not seem to be required during the actual LIF-induced rapid differentiation of the M1/WT cells, its activity still seems to be needed to enable a M1 cell population to become rapidly responsive to LIF and IL-6. One possibility is that during passaging M1/WT cells are differentiated, to some extent at least, in a CSF-1R kinase activity-dependent manner, which may or may not require the action of endogenous CSF-1. We therefore sought evidence for differences in LIF-dependent signaling between the M1 parental and M1/WT populations. It

could be that there might be heightened signal transduction through the LIF receptor due to, for example, increased LIF receptor expression in the M1/WT cells. The activation of the transcription factor signal transducer and activator of transcription 3 (STAT3) has been implicated in myeloid differentiation, including in LIF-induced and IL-6-induced macrophage differentiation in M1 cells and in general in the action of LIF (25-29). We see in Fig. 4A that in fact M1/WT cells express less STAT3 protein, and after LIF treatment, the levels of phosphorylated STAT3 are lower than in the M1 parental cells. In addition, as Fig. 4B shows, there were striking differences in the global acute tyrosine phosphorylation pattern before and after LIF addition for the two cell populations, indicating a qualitative difference in the LIF-dependent signaling cascades used rather than a potentiation in the signaling via the LIF receptor in the M1/WT cells. For example, an ~100 kDa tyrosine-phosphorylated protein present in lysates of M1 parental cells, irrespective of whether the cells had been stimulated with LIF, was not observed in lysates of M1/WT cells (Fig. 4B). Conversely, an ~70 kDa tyrosine-phosphorylated protein, which underwent further tyrosine phosphorylation in response to LIF, was present in lysates of M1/WT cells but was not detected in lysates of the M1 parental cells (Fig. 4B).

The lack of CSF-1R in the M1 parental lysates can be seen in Fig. 4C as can the lack of disappearance of the CSF-1R in the M1/WT cells, the latter observation consistent with the lack of its tyrosine phosphorylation and internalization mentioned earlier, supporting the conclusion above that LIF does not act via CSF-1R activation in the M1/WT cells.

LIF-Dependent and CSF-1-Dependent Signal Transduction

Because the evidence thus far points to CSF-1R-independent signaling to explain the rapid macrophage differentiation in response to LIF (and IL-6), we compared LIF-dependent and CSF-1-dependent signal transduction. We analyzed the activation of extracellular signal-regulated kinase (ERK), Akt, and STAT3 in M1/WT cells in response to LIF, CSF-1, and their combination.

ERK Activation. Inhibition of the MEK/ERK pathway blocks both CSF-1-dependent differentiation in M1/WT cells (22) and IL-6-dependent M1 cell differentiation (30). We assessed therefore whether there might be an additive effect on this pathway when CSF-1 and LIF were coadded. As can be seen in Fig. 5A, CSF-1 induced a sustained ERK1/2 activation; there was a weak, if any, ERK1/2 activation by LIF itself and no evidence for an additive effect.

Akt Activation. When Akt activation was monitored, CSF-1 gave a transient stimulation but LIF did not; when added together, it seemed that there might be a transient potentiation at ~4 h (Fig. 5B).

STAT3 Activation. In contrast to ERK activation, as we saw in Fig. 4A, LIF induced a strong and sustained STAT3 activation; however, CSF-1 gave only a delayed response suggesting perhaps an indirect effect (Fig. 5C). There was no evidence for any additive effect for the two stimuli.

The delayed STAT3 phosphorylation observed in CSF-1-treated M1/WT cells (Fig. 5C) suggested that an endogenous mediator may be accumulating in the culture medium with

possible implications also for the control of the CSF-1-induced differentiation. As Fig. 5D shows, conditioned medium from CSF-1-treated M1/WT cells, but not from CSF-1-treated parental cells, was able to induce rapid STAT3 tyrosine phosphorylation. Because (a) CSF-1 can stimulate monocytes/macrophages to produce IL-10 (31), (b) IL-6 induces IL-10 production during M1 cell macrophage differentiation (32), (c) STAT3 activation is central to the action of IL-10 on monocytes/macrophages, and (d) IL-10 can promote macrophage lineage differentiation in primary cells (33), we explored whether endogenous IL-10 might be contributing to M1/WT differentiation. CSF-1 can indeed induce IL-10 secretion in M1/WT cells but not in the M1 parental counterpart, and transient IL-10 mRNA expression was noted after 2 to 4 h in the CSF-1-treated M1/WT cells (data not shown). However, for M1/WT cells, IL-10 did not induce differentiation when added alone (as measured by reduced cell number) and did not enhance the differentiation induced by CSF-1, and neutralizing antibody to the IL-10 receptor did not reduce the CSF-1-induced macrophage differentiation (data not shown), suggesting that endogenous IL-10 is unlikely to be contributing to the CSF-1-induced differentiation, although likely to be activating STAT3.

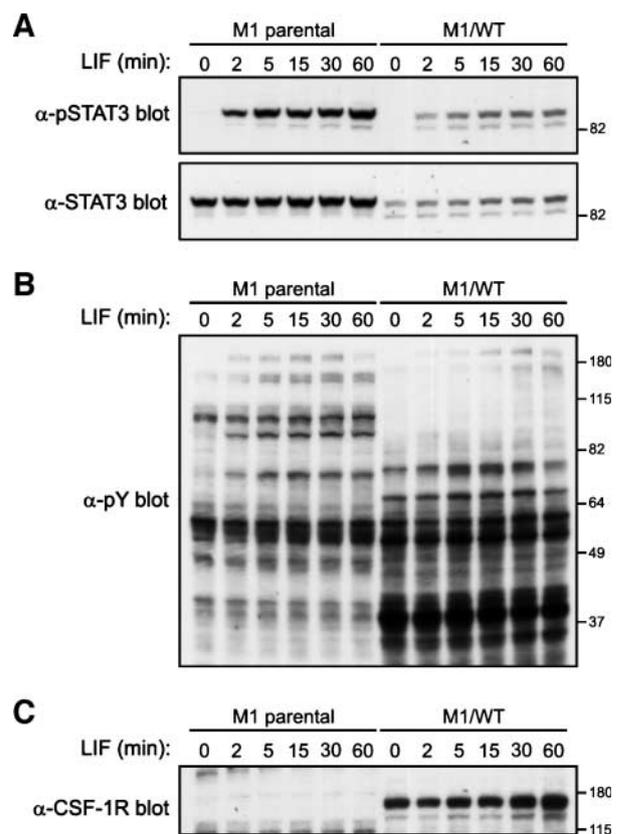


FIGURE 4. Analysis of LIF-induced signal transduction in parental M1 cells and M1 cells expressing CSF-1R. M1 parental and M1/WT cells were cultured in the presence of 1.0% newborn calf serum for 60 min and then stimulated with LIF (10 ng/mL). The cells were collected at the indicated time points and lysed, and aliquots of the cell lysates were subjected to Western blotting with anti-phosphorylated STAT3 and anti-STAT3 antibodies (A), an anti-phosphotyrosine (4G10) antibody (B), and an anti-CSF-1R antibody (C).

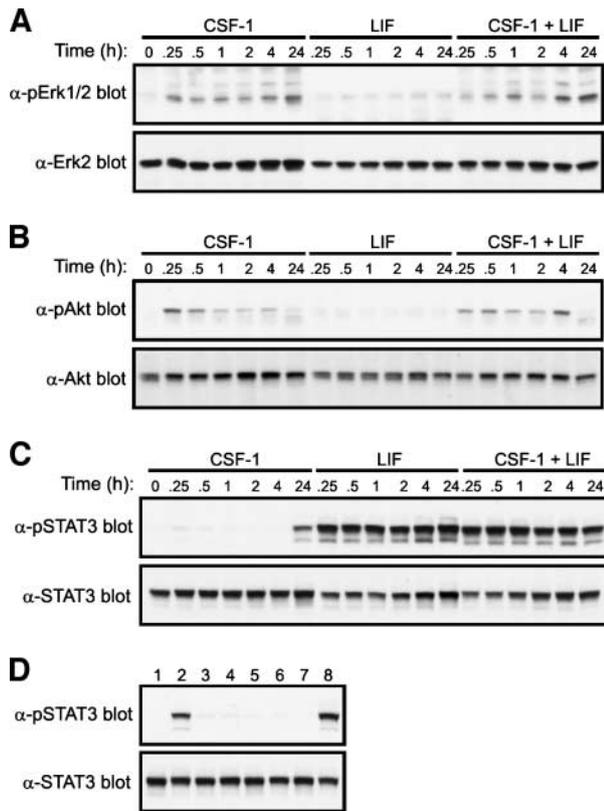


FIGURE 5. Analysis of CSF-1–induced and LIF-induced signal transduction in M1 cells expressing CSF-1R. **A to C.** M1/WT cells were cultured in the presence of 1.0% newborn calf serum for 60 min and then stimulated with CSF-1 (5×10^3 units/mL), LIF (10 ng/mL), or both CSF-1 and LIF. The cells were collected at the indicated time points and lysed, and aliquots of the cell lysates were subjected to Western blotting with the indicated antibodies. The data presented are representative of four independent experiments. **D.** M1/WT cells (1×10^6 /mL) were cultured in the presence of 1.0% newborn calf serum for 60 min and then untreated (lane 1) or stimulated for 30 min with conditioned medium from M1/WT cells grown in the presence (lane 2) and absence (lane 3) of CSF-1 (5×10^3 units/mL) for 24 h, conditioned medium from M1/WT cells grown in the absence of CSF-1 for 24 h and then supplemented with CSF-1 (5×10^3 units/mL; lane 4), conditioned medium from M1 parental cells grown in the presence (lane 5) and absence (lane 6) of CSF-1, CSF-1 (5×10^3 units/mL; lane 7), and LIF (10 ng/mL; lane 8). The cells were then collected and lysed, and aliquots of the cell lysates were subjected to Western blotting with the indicated antibodies. The data presented are representative of three independent experiments.

LIF-Induced and IL-6–Induced Differentiation in the Presence of CSF-1

We reasoned that if the CSF-1R–dependent macrophage differentiation induced by LIF was not dependent on the secretion of endogenous CSF-1 (Fig. 3B and C) and given the differences in the downstream signaling cascades for LIF and CSF-1 (Fig. 5A–C), then coaddition of CSF-1, even at optimal levels for differentiation, might lead to further differentiation. CSF-1 induces maximal differentiation of M1/WT cells at concentrations ~ 500 units/mL with no further differentiation even if 10^4 units/mL CSF-1 was added (data not shown). We found that coaddition of LIF and an optimal CSF-1 concentration resulted in further rapid differentiation of M1/WT cells as measured by macrophage-like morphology (Fig. 6A), forward

and side scatter profiles (Fig. 6B), cell number (Fig. 6C), and both Mac-1 expression per cell (Fig. 6D) and also the proportion of Mac-1–positive cells (data not shown); similar observations were made for coaddition of IL-6 and CSF-1 (Fig. 6D). The observed added effects occurred if LIF concentrations were suboptimal (0.3 ng/mL; Fig. 6A, B, and D) or optimal (10 ng/mL; Fig. 6C). The potentiation was noted if suboptimal concentrations of both LIF and CSF-1 were used and GW2580 inhibited the potentiation by CSF-1 but not by LIF (data not shown). No potentiation of the coaddition over 3 days was noted for the corresponding experiments with the parental M1 cells. It can be observed for M1/WT cells that LIF induces more growth suppression (Figs. 2A and 6C) and apoptosis (Figs. 2C and 6E) than CSF-1. CSF-1 did not increase the extent of apoptosis resulting from the action of LIF (Fig. 6E).

Because LIF and IL-6 can trigger STAT3 phosphorylation in M1 cells, endogenous LIF and/or IL-6 could be responsible for the secreted activity noted above for the CSF-1–treated M1/WT cells (Fig. 5D); however, because coaddition of optimal concentrations of LIF (10 ng/mL) or IL-6 (100 ng/mL) with CSF-1 leads to enhanced macrophage-like differentiation in the M1/WT cells (Fig. 6), it is unlikely that endogenous LIF or IL-6 would be contributing to this potentiation.

Discussion

We showed previously that M1/WT cells differentiate rapidly into macrophage-type cells in response to CSF-1 (14, 21); we have now shown by several criteria (morphology, proliferation rate, apoptosis, and Mac-1 expression) that the M1/WT cells were also more rapidly converted by LIF and IL-6 into a macrophage phenotype than M1 cells not expressing the CSF-1R. In addition, an additive effect was noted if LIF and IL-6 were added in the presence of an optimal CSF-1 concentration. Using neutralizing antibody to the CSF-1R and a CSF-1R kinase antagonist, we could find no evidence for the action of endogenous CSF-1 to be mediating the enhanced differentiation of the M1/WT cells by LIF; this conclusion is consistent with the additive effect observed between LIF and optimal exogenous CSF-1. Supporting observations on the lack of involvement of endogenous CSF-1 have been reported by Aperlo et al. (11) using anti-CSF-1 blockade during studies of LIF-mediated differentiation in a M1 cell population. Our new findings with LIF and IL-6 mirror what we found with agents that raise intracellular cyclic AMP (22), indicating that they are not confined to the LIF and IL-6 cytokine family acting via the gp130 receptor subunit.

There is strong evidence that STAT3 activation is critical for LIF-induced and IL-6–induced macrophage differentiation in M1 cells (26–29). Based on the observation that, for the “synergistic” response between LIF and CSF-1 for M1 differentiation, the two cytokines can be added sequentially, it was concluded that the respective cellular responses were independent but complementary (11). We showed above that this independence of the respective responses is reflected at the level of signal transduction with CSF-1, in contrast to LIF, able to stimulate ERK and Akt activation in M1/WT cells; conversely, LIF induced a rapid STAT3 phosphorylation with

CSF-1 only able to do this in a delayed manner, suggesting the possible involvement of an endogenous mediator in the STAT3 phosphorylation. The identity of this putative “mediator” and its role, if any, in the action of CSF-1 is unknown. As discussed earlier, this mediator is unlikely to be LIF, IL-6, or IL-10. In contrast to what we found previously with agents that can raise cyclic AMP (22), there was no potentiation by LIF of the CSF-1–stimulated ERK activity, implicated in the differentiation response of the M1/WT cells to CSF-1; also following coaddition of CSF-1 and LIF, we found no evidence for a potentiation of STAT3 phosphorylation. Thus, further mechanistic studies are required to explain the enhanced terminal differentiation by LIF (and IL-6) of M1/WT cells, either in the presence or absence of CSF-1. It is likely that the macrophage phenotypes generated by LIF and CSF-1, respectively, will differ; in this context, we observed that only LIF induces detectable apoptosis in M1/WT cells over the short time scale studied (Figs. 2C and 6E).

Paradoxically, although CSF-1R kinase activity did not seem to be required during the actual LIF-induced rapid differentiation of the M1/WT cells, its activity still seems to be needed to enable a M1 cell population to become rapidly responsive to LIF and IL-6 as judged by the poor response of M1/807 cells to the cytokines. Our interpretation is that during passaging M1/WT cells are differentiated, to some extent at least, in a CSF-1R kinase activity-dependent manner, which may or may not require the action of endogenous CSF-1. Tyrosine phosphorylation, and therefore presumably activation, of CSF-1R was noted in untreated M1/WT cells (14) and others have suggested that overexpression of tyrosine kinase receptors, including CSF-1R, can lead to a constitutive signal in the absence of ligand due to an increase in the total number of ligand-independent activated receptors in unstimulated cells (34). In support of our concept of a difference between the two cell types as a result of passaging, the data in Fig. 4 show that the pattern of LIF signaling in the M1/WT cells differs from that observed in

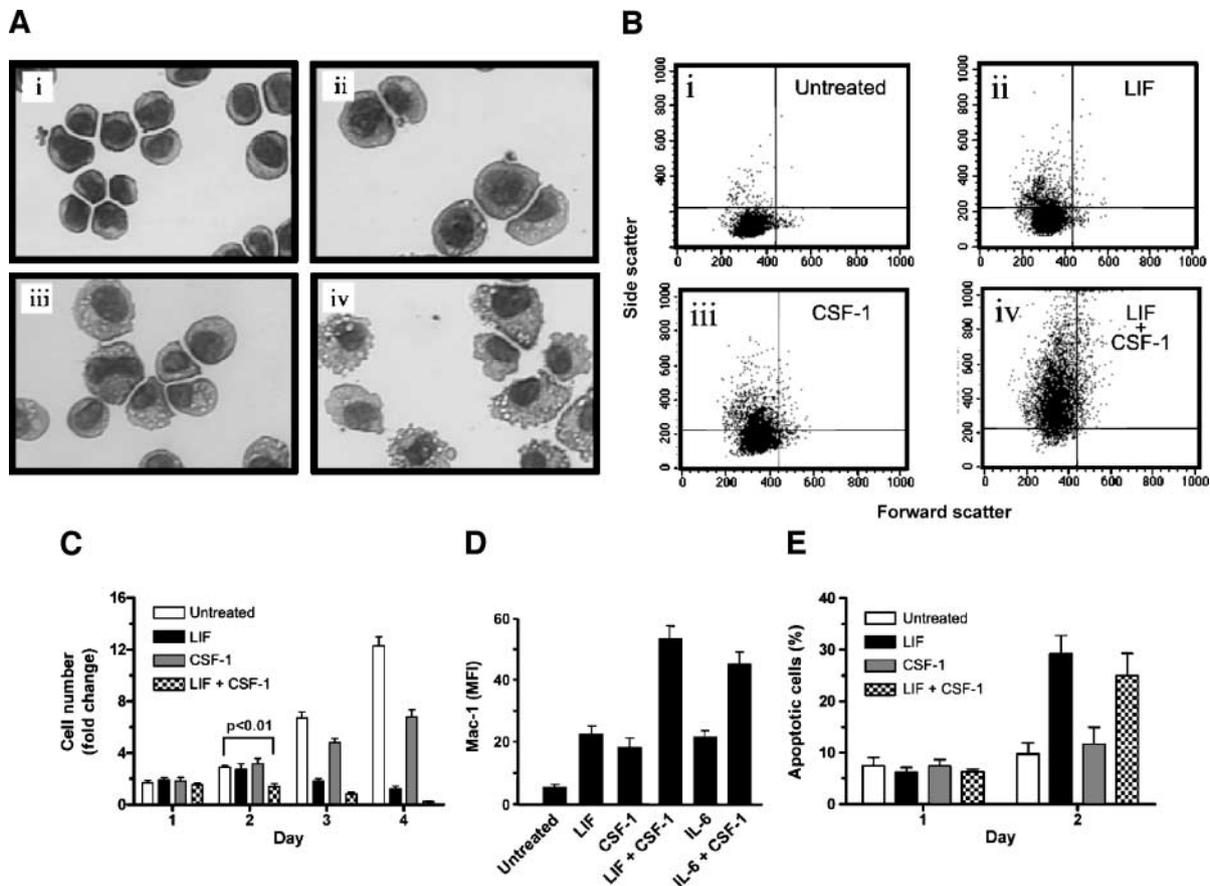


FIGURE 6. LIF-induced and IL-6–induced macrophage differentiation in M1 cells expressing CSF-1R in the presence of an optimal CSF-1 concentration. **A** and **B.** M1/WT cells (2×10^5 /mL) were either untreated or treated with CSF-1 (5×10^3 units/mL) or LIF (0.3 ng/mL) alone or in combination. After 2 d, cells were harvested and (**A**) stained by the Diff-Quik method ($\times 400$ magnification; *i*, untreated; *ii*, LIF; *iii*, CSF-1; *iv*, LIF + CSF-1) or (**B**) forward and side scatter profiles were analyzed by flow cytometry. This experiment was repeated seven times. **C.** M1/WT cells (7×10^4 /mL) were cultured in the presence or absence of LIF (10 ng/mL), CSF-1 (5×10^3 units/mL), or both LIF and CSF-1 for the indicated number of days. Viable cell numbers were then enumerated and expressed as the fold change in cell number over the number of cells seeded at day 0. Columns, mean of three independent experiments (triplicate cultures); bars, SE. *P* value was determined using the Student's *t* test. **D.** M1/WT cells (2×10^5 /mL) were cultured for 2 d in the presence or absence of LIF (0.3 ng/mL), CSF-1 (5×10^3 units/mL), or IL-6 (10 ng/mL) alone, or with LIF or IL-6 in the presence of CSF-1. Mac-1 levels were then quantified by flow cytometry and expressed as mean fluorescence intensity (MFI). Columns, mean of six experiments (triplicate cultures); bars, SE. **E.** M1/WT cells (7×10^4 /mL) were cultured in the presence or absence of LIF (10 ng/mL), CSF-1 (5×10^3 units/mL), or both LIF and CSF-1 for the indicated number of days. The percentage of apoptotic cells (propidium iodide⁺ and/or Annexin V⁺) was measured. Columns, mean of three independent experiments (triplicate cultures); bars, SE.

the M1 cells and the levels of phosphorylated STAT3 in the LIF-treated M1/WT cells were in fact lower than in the correspondingly treated M1 parental population. Presumably, the lower levels of phosphorylated STAT3 in LIF-treated M1/WT cells are sufficient for their rapid differentiation. Some additional support for our interpretation is the slight morphology difference between the passaged M1/WT and M1 parental cells before CSF-1 or LIF addition (Fig. 1) and the fact that the M1/WT cells grow more slowly in serum than the M1 parental cells (Fig. 2). As mentioned, up-regulation of CSF-1R expression has been noted as part of the differentiation-associated changes following LIF and IL-6 addition to M1 cell populations (11, 12) and it was concluded that LIF in this way prepares the cells to respond optimally to CSF-1 (11). What we have shown, by studying M1 cells engineered to express CSF-1R, is that LIF and IL-6 can not only induce CSF-1R expression as part of the macrophage differentiation program, thereby enabling a cell to respond to CSF-1, but also cross-talk with and therefore potentiate CSF-1R-dependent signaling. We suggest that over time during growth activated CSF-1R can initiate a differentiation program in M1/WT cells, thereby reciprocally preparing a cell to respond rapidly and optimally to agents such as LIF and IL-6. The short circuiting of the terminal macrophage differentiation program in M1/WT cells in response to LIF and IL-6 most likely mimics the completion of the normal LIF-induced and IL-6-induced pathways in M1 cells, with involvement of CSF-1R activation, possibly by endogenous CSF-1. Our findings with the M1/WT cells would not seem to be due simply to artifactual overexpression of CSF-1R because, as judged by mAb staining and flow cytometry, its levels in the M1/WT cells are slightly less than those in murine peritoneal macrophages and *in vitro*-derived bone marrow macrophages (data not shown).

We also propose that CSF-1R expression and subsequent activation may spontaneously occur to some extent over time during normal M1 cell growth. In this context, M1 populations have been reported to contain cells with CSF-1R on their surface (14, 20). As mentioned, we took the precaution to remove CSF-1R⁺ cells from our starting M1 populations so that the presence of such cells would not complicate our studies (14). A corollary of this proposal is that any cells with CSF-1R at a sufficient level in M1 populations used in the literature for macrophage differentiation analysis will respond more rapidly and dramatically to LIF in the short term than their counterparts with no CSF-1R or at levels below a presumed critical threshold level. If this were so, differences in the rate of differentiation might be expected to be observed with various populations of "M1 cells" used in the literature, which are dependent on the proportion of cells expressing sufficient CSF-1R. There is support for this notion as several reports indicate that at the clonal level M1 cells show all or none responsiveness to LIF (8) and that LIF and IL-6 can induce differentiation more rapidly in M1 cell populations (measured, for example, by morphology, growth suppression, and Mac-1 expression; refs. 8, 11, 17, 23) than what we found with our so-called M1 parental cells (i.e., depleted of CSF-1R⁺ cells). We suggest in studies of M1 cell differentiation that the proportion of CSF-1R⁺ cells in the starting population should be taken into account.

Other approaches have resulted in the induction of M1 cell macrophage differentiation program(s), which also includes the

early appearance of CSF-1R. Two such examples are the constitutive expression of the transcription factors *ets-2* (15) and Wilms' tumor suppressor gene (16). Addition of LIF to both of these modified M1 populations also resulted in rapid and terminal macrophage differentiation, growth suppression, and cell death when compared with that seen in the unmodified starting population. Based on the findings with the Wilms' tumor isoform expression system, Smith et al. (16) concluded that a second signal evoked by LIF is required to complete the program of macrophage differentiation. As an extension of this notion, we are now proposing that this second signal involves CSF-1R-dependent pathways as discussed above. Combinations of LIF, IL-6, and granulocyte CSF have been reported to enhance M1 cell differentiation as an "integrated biological activity" (8, 17); also intriguingly, a diverse range of agents all induces macrophage differentiation in M1 cell populations and usually not differentiation along other cell lineages (2, 12, 17, 18, 23). We suggest that all of these prior literature observations listed above on the differentiation of M1 cell populations (8, 11, 12, 16-19) can be explained by a unifying concept that for the various inducers of M1 cell differentiation CSF-1R-dependent signaling is a critical, common, and relatively early differentiation event. There is some evidence for specificity for CSF-1R-dependent signaling in that ectopic GM-CSF receptor expression in M1 cell populations did not potentiate the macrophage differentiation due to LIF (19).

A major effect of CSF-1 on human bone marrow cells and monocytes *in vitro*, as well as on blast cells of acute myeloid leukemia, is differentiation rather than proliferation (13, 35); we have reported that during murine osteoclastogenesis CSF-1 seems to provide a differentiation signal (36). In addition, there are *in vivo* and *in vitro* data indicating that CSF-1 can negatively regulate both the maintenance and commitment of multipotent precursor cells into other lineages by promoting macrophage lineage development (37, 38). CSF-1R ectopic expression in M1 cells provides a model illustrating that the macrophage differentiation program is a multistep process that can be arrested at intermediate stages and uncoupled from terminal differentiation usually associated with growth suppression and cell death. As well as mimicking what happens normally in M1 cells over time in culture, we also propose that this ectopic CSF-1R expression mimics what happens during normal macrophage lineage development (6, 7). If CSF-1R kinase expression and/or activity is compromised during or following this period, then normal lineage maturation would not occur, thereby favoring the maintenance of an undifferentiated state or even a leukemic phenotype in the presence of other proleukemic cellular changes. Some findings with normal hemopoietic cell populations that support this M1 model for macrophage lineage commitment are that (a) up-regulation of CSF-1R expression by IL-6 is important in the control of the differentiation of human myeloid populations preferentially along the macrophage lineage (39, 40); (b) LIF can synergize with growth factors, including CSF-1, in hemopoiesis (41); (c) up-regulation of CSF-1R levels favors myeloid over lymphoid differentiation in cytokine-treated hemopoietic progenitor cells (3); and (d) monocytopenia is controlled by a circuitry involving sequentially microRNAs 17.5p-20a-106a, the transcription factor acute myeloid leukemia-1, and CSF-1R (7).

Materials and Methods

Cells

M1 murine myeloid cells (8, 17) were a gift from N. Nicola (Walter and Eliza Hall Institute, Parkville, Victoria, Australia). Before experimentation, they were depleted of CSF-1R-positive cells as before (14); the depleted population, referred to as parental M1 cells, was transfected with normal or "WT" CSF-1R to give rise to the so-called M1/WT cells, as published (14). The parental M1 cells were also transfected with *c-fms* containing a tyrosine to phenylalanine mutation at position 807 (M1/807) as follows. Briefly, WT *c-fms* cDNA (obtained from D. Hume, University of Queensland, Brisbane, Queensland, Australia) was ligated into the pEF-BOS expression vector (42) and used as a template for subsequent mutational PCR. Mutation of Tyr⁸⁰⁷ to phenylalanine was generated by PCR using mutagenic primers, which substituted the 807 codon from TAC (Y) to TTC (F). All PCR fragments were generated using the Expand High Fidelity PCR System (Boehringer Mannheim). The PCR fragments containing the Y807F mutation were then cloned into the WT *c-fms* gene and sequenced to ensure the absence of unwanted mutations. The expression vector was introduced into parental M1 cells as described previously (14). As before (14, 21, 22, 43, 44), after stable transfection, M1 clones were pooled and then sorted by fluorescence-activated cell sorting to achieve a mixed population of cells with similar receptor number. All M1 cell populations were maintained in DMEM (CSL) containing 10% newborn calf serum (CSL) at 37°C in 5% humidified CO₂.

Cell Morphology and Proliferation

Cells were washed in PBS without Ca²⁺ (PD) and resuspended at 1 × 10⁶/mL; 50 μL were then loaded into a cytospin chamber containing a glass microscope slide and centrifuged at 800 rpm for 3 min, and the slides were air dried before staining by the Diff-Quik method. Images were acquired with a Sony Digital Hyper HAD color video camera (Sony Corp.) and Leica Q500MC Windows software (Leica). Viable cell number was measured by trypan blue exclusion with a hemocytometer.

Mac-1 and CSF-1R Staining

Cells (2 × 10⁵) were washed twice in ice-cold PD, resuspended in either 50 μL rat anti-Mac-1 mAb, anti-CSF-1R mAb (AFS-98; ref. 45), or isotype-matched control mAb, and left on ice for 1 h. Cells were washed thrice in ice-cold PD and then resuspended in FITC-conjugated anti-rat IgG and left on ice for a further 30 min. Cells were then washed twice in ice-cold PD and resuspended in 300 μL PD with propidium iodide. Fluorescence was measured using a FACSCalibur flow cytometer (Becton Dickinson). Acquisition was restricted to 10,000 events for each sample and Mac-1 and CSF-1R expression was calculated by subtracting the isotype-matched control value from the Mac-1 and CSF-1R values. Data were analyzed by using CellQuest version 3.0.1.

Forward and Side Scatter Profiles

Forward and side light scatter profiles of the cells were measured using the FACSCalibur flow cytometer (14). Quadrants were set using M1/WT cells cultured in newborn calf serum alone

so that 95% of cells lay in the lower left quadrant. Cells outside this quadrant were considered to have undergone differentiation.

Signal Transduction

Cells were collected at the required time points by centrifugation, quickly washed with ice-cold PD, and then lysed in NP40 lysis buffer [20 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 2 mmol/L EDTA, 1.0% NP40, 10% glycerol, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium molybdate, 10 mmol/L NaF, 10 mmol/L β-glycerophosphate, Complete protease inhibitors] for 30 min on ice. Lysates were clarified by centrifugation at 13,000 × *g* for 10 min at 4°C and protein concentrations were measured with a Bio-Rad protein assay kit. Western blotting of cell lysates was done by standard techniques.

Cytokine Levels

The levels of IL-10 in tissue culture supernatants were measured using a mouse OptEIA ELISA kit (BD PharMingen) according to the manufacturer's instructions. IL-10 mRNA levels were measured by quantitative real-time PCR using an ABI PRISM 7900HT sequence detection system and predeveloped Taqman probe/primer combinations for IL-10 and 18S rRNA.

Reagents

The following were obtained as gifts: recombinant human CSF-1 (Chiron), recombinant human LIF (N. Nicola), recombinant human IL-6 (R. Simpson, Ludwig Institute for Cancer Research, Parkville, Victoria, Australia), and anti-CSF-1R rat mAb (AFS-98; S-I. Nishikawa, Kyoto University, Kyoto, Japan; ref. 45). The following were from commercial sources: anti-Mac-1 hybridoma cells (American Type Culture Collection), rat anti-mouse IgG2b (BD Biosciences), FITC-conjugated anti-rat IgG (Chemicon Australia), and GW2580 (Calbiochem, EMD Chemicals, Inc.). The anti-phosphorylated ERK1/2, anti-phosphorylated Akt, anti-Akt, anti-phosphorylated STAT3, and anti-STAT3 antibodies were from Cell Signaling Technology, Inc., whereas the anti-ERK2 and anti-phosphotyrosine (4G10) antibodies were from Santa Cruz Biotechnology, Inc. and Millipore, respectively.

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