Macrophage Proliferation Is Regulated through CSF-1 Receptor Tyrosines 544, 559, and 807* □

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Colony-stimulating factor-1 (CSF-1)-stimulated CSF-1 receptor (CSF-1R) tyrosine phosphorylation initiates survival, proliferation, and differentiation signaling pathways in macrophages. Either activation loop Y807F or juxtamembrane domain (JMD) Y559F mutations severely compromise CSF-1-regulated proliferation and differentiation. YEF, a CSF-1R in which all eight tyrosines phosphorylated in the activated receptor were mutated to phenylalanine, lacks in vitro kinase activity and in vivo CSF-1-regulated tyrosine phosphorylation. The addition of Tyr-807 alone to the YEF backbone (Y807AB) led to CSF-1-independent but receptor kinase-dependent proliferation, without detectable activation loop Tyr-807 phosphorylation. The addition of Tyr-559 alone (Y559AB) supported a low level of CSF-1-independent proliferation that was slightly enhanced by CSF-1, indicating that Tyr-559 has a positive Tyr-807-independent effect. Consistent with the postulated autoinhibitory role of the JMD Tyr-559 and its relief by ligand-induced Tyr-559 phosphorylation, the addition of Tyr-559 to the Y807AB background suppressed proliferation in the absence of CSF-1, but restored most of the CSF-1-stimulated proliferation. Full restoration of kinase activation and proliferation required the additional add back of JMD Tyr-544. Inhibitor experiments indicate that the constitutive proliferation of Y807AB macrophages is mediated by the phosphatidylinositol 3-kinase (PI3K) and ERK1/2 pathways, whereas proliferation of WT and Y559,807AB macrophages is, in addition, contributed to by Src family kinase (SFK)-dependent pathways. Thus Tyr-807 confers sufficient kinase activity for strong CSF-1-independent proliferation, whereas Tyr-559 maintains the receptor in an inactive state. Tyr-559 phosphorylation releases this restraint and may also contribute to the CSF-1-regulated proliferative response by activating Src family kinase.

Colony-stimulating factor-1 (CSF-1) regulates the survival, proliferation, and differentiation of macrophages and osteoclasts via the CSF-1 receptor (CSF-1R) (reviewed in Refs. 1 and 2), a 165-kDa glycoprotein receptor tyrosine kinase encoded by the c-fms proto-oncogene (3). The CSF-1R is a member of the platelet-derived growth factor receptor (PDGFR) family of class III receptor tyrosine kinases that includes PDGFRα/β, stem cell factor receptor (c-Kit), and Flt3/Fk1 (reviewed in Ref. 4). The two known CSF-1R ligands, CSF-1 and interleukin-34 (5), are both divalent and activate signaling through the receptor in a similar fashion, but differ in their developmental and tissue-specific expression patterns (6). Recent studies have demonstrated the importance of CSF-1R regulation of macrophages and osteoclasts in inflammatory disease (2) and of tumor-associated macrophages in the enhancement of tumor progression and metastasis (7–9).

Members of the PDGFR family possess an extracellular domain of five immunoglobulin domain loops (D1–D5), a transmembrane domain, a cytoplasmic juxtamembrane domain (JMD), a split cytoplasmic kinase domain composed of an ATP-binding domain, a kinase insert domain, and a major kinase domain and a C-terminal tail (4). Ligand-induced mouse CSF-1R extracellular domain dimerization results in the phosphorylation of six cytoplasmic domain tyrosine residues, tyrosines 559, 697, 706, 721, 807, and 974, and the phosphorylation of Tyr-544 and Tyr-921 has been reported for an activated oncogenic form of the receptor (10, 11). Phosphorylation of the majority of these tyrosines creates docking sites for downstream signaling molecules that contain phosphotyrosine-binding domains (reviewed in Refs. 1, 12, and 13).

Receptor tyrosine kinase tyrosine phosphorylation is also involved in ligand-induced receptor activation. Studies of the PDGF receptor family (14–17) and other receptor tyrosine kinases (18) (reviewed in Ref. 19) indicate that the JMD regulates receptor activation. In the unliganded state, the JMD plays an important autoinhibitory role through its insertion between...
the kinase N- and C-lobes to sterically lock the activation loop (AL) in its inactive conformation. Ligand binding relieves this inhibition by phosphorylation of the JMD tyrosines. In the case of the activated stem cell factor receptor, phosphorylation of the JMD Tyr-567 and Tyr-569 is responsible, permitting the active conformation of the AL (16).

Unlike the other PDGFR family members, there is a sole conserved tyrosine (559) in the switch region of the CSF-1R, corresponding to Tyr-567 of c-Kit. Similar to the stem cell factor receptor Phe-567/Phe-569 mutant, the CSF-1R Phe-559 mutation significantly reduces in vitro kinase activity (20) and markedly inhibits ligand-stimulated tyrosine phosphorylation in vivo (20–22). Consistent with the role of Tyr-559 as a switch, it is the first tyrosine to be phosphorylated in the activation of the wild type CSF-1R (23). However, apart from its critical role in CSF-1R activation, phosphorylation of Tyr-559 is both necessary (21, 24) and sufficient (23) for activation of an SFK/c-Cbl/CSF-1R ubiquitination pathway that on the one hand, permits full receptor tyrosine phosphorylation (23) and on the other hand, mediates ligand-induced receptor internalization and degradation (21, 23) that attenuate proliferation signaling (25).

Phosphorylation of AL tyrosines has been shown to increase regional hydrophilicity, extending the loop and altering the spatial relationship between the ATP-binding domain and major kinase domain (26–28). No protein has been identified to bind to the phosphorylated AL Tyr-807 site of the CSF-1R (reviewed in Ref. 13). In macrophages, consistent with the critical roles of the JMD Tyr-559 and AL Tyr-807 in the activation and function of the receptor, the Phe-559 and Phe-807–mutant receptors significantly compromise CSF-1R-regulated proliferation and differentiation (20, 22).

To study the structure-function relationships of the CSF-1R in the macrophage, we created a cloned conditional CSF-1R-deficient mouse bone marrow macrophage cell line, MacCsf1r−/− (M−/−), which, when transduced with the WT CSF-1R, exhibits the CSF-1-dependent survival, proliferation, morphological, and differentiation responses of the primary bone marrow–derived macrophages from which it was derived (20). In the present study, to further understand the function of the CSF-1R tyrosines, we have added back tyrosines to a receptor backbone (YEF) and studied the CSF-1 response in macrophages of the various M−/−, YEF:YAB lines, focusing on those containing JMD Tyr-559 and AL Tyr-807 that play important roles in CSF-1R activation and CSF-1-regulated cell proliferation in macrophages.

**EXPERIMENTAL PROCEDURES**

*Reagents*—The anti-mouse CSF-1R peptide (962GDIAQ-PLLQPNNYQ<sup>976</sup>) antiserum (carboxy terminus) and the anti-pY559 CSF-1R peptide antibody (EGNSpYT-FIDPTQLPYNEK<sup>977</sup>) were raised in rabbits and affinity-purified against their corresponding peptides. The anti-whole CSF-1R antiserum was raised in goat as described earlier (29). Anti-phosphotyrosine-Ser (PY100), anti-human CSF-1R phospho-Tyr-809 antibody, anti-phospho-p44/42 MAPK, anti-phospho-p38 MAPK, anti-phospho-Ser-473 Akt, anti-phospho-Thr-308 Akt, anti-p44/42 MAPK, anti-p38 MAPK, anti-phospho-Src family (Tyr-416), and anti-phospho-Src (Tyr-527) were from Cell Signaling Technology (Beverly, MA). Anti-Hck and anti-phospho-Hck (Tyr-411) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Human recombinant CSF-1 was a gift from Chiron Corp., Emeryville, CA. Polyclonal Tyr-416, and anti-phospho-Src (Tyr-527) were from Cell Signal-RESEARCH. MAPK, anti-phospho-Ser-473 Akt, anti-phospho-Thr-308 Akt, and anti-phospho-Src (Tyr-527) were from Cell Signaling Technology (Beverly, MA). Anti-Hck and anti-phospho-Hck (Tyr-411) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Human recombinant CSF-1 was a gift from Chiron Corp., Emeryville, CA. PolyGluAlaTyr (polyEAY) and 7-aminoactinomycin D were from Sigma. EZ-Link Sulfo-NHS-LC-CL-Biotin and EZ-Link sulfo-NHS-biotin and bis(sulfosuccinimidyl) substrate (BS<sup>3</sup>) were from Pierce. SU6656, LY294002, JNK inhibitor (SP600125, catalog number 420119), and p38 MAPK inhibitor (catalog number 506126) were purchased from Calbiochem. PD98058 was purchased from BioMol (Plymouth Meeting, PA). PLX3397 and PLX5622 were gifts from Plexxikon Inc. (Berkeley, CA). Trans<sup>35</sup>S-LABEL<sup>TM</sup> No-Thaw metabolic labeling reagent was purchased from MP Biochemicals (Solon, OH). The derivation of the cloned MacCsf1r−/− (M−/−) and MacCsf1r+/+/ (M+/+) cell lines, the preparation of granulocyte-macrophage conditioned medium, and the nature and origin of the c-fms c-DNA and retroviral vectors used to prepare the retroviruses used were described previously (20).

*Site-directed Mutagenesis, Retroviral Infection, and Analysis of MacCsf1r−/− Cells*—The pZen113xNc-FMS YQF/Y559F/Y974F and WT c-fms plasmids were used to produce CSF-1 YEF (20) in which eight tyrosines (544, 559, 697, 706, 821, 827, 921, and 974) were replaced by phenylalanine. Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Using CSF-1R YEF as backbone, single, double, and triple tyrosine add-back mutants were generated. All introduced mutants were confirmed by sequencing. The coding regions of the mutant CSF-1R cDNAs were inserted into the MSCV-ires-GFP vector (30) at the EcoRI/XhoI site upstream of the internal ribosomal entry site (ires) driving expression of GFP. Retroviral transduction of the cloned MacCsf1r−/− (M−/−) macrophage cell line and the selection of lines with cell surface CSF-1R expression levels approximating those of WT (M+/+) macrophages was carried out by cell sorting, as described previously (20). Cells cultured in GM-CSF or CSF-1 for 3 months maintained stable CSF-1R expression, and cells thawed for experiments were passaged for no longer than 2 months. Cell survival, proliferation, differentiation, and CSF-1 stimulation were carried out as described (20). In one experiment, cells were incubated with or without CSF-1 for 2 h at 4 °C in the presence of 400 μM Na<sub>3</sub>VO<sub>4</sub> under previously established conditions optimizing in vivo CSF-1R tyrosine phosphorylation (31).

*In Vitro CSF-1R Kinase and Autophosphorylation Assays, Immunoprecipitation, and Western Blotting*—In vitro CSF-1R autophosphorylation and kinase assays with polyEAY as substrate are described in detail elsewhere (20). Immunoprecipitations, SDS-PAGE, and Western blot analyses were performed as described previously (31, 32). Blotted membranes were incubated with HRP substrate (Millipore Corp., Billerica, MA), and the chemiluminescent signals were recorded by an Image Reader LAS-3000 (Fuji Film, Tokyo, Japan) and analyzed with the software ImageGauge from Fuji Film. Assays involving SDS-PAGE contained a M−/− WT lysate control and were normalized by comparison with the activity of the stable BAC1.2F5 macrophage (33) lysate stored at −80 °C.
5-Ethynyl-2'-deoxyuridine (EdU) Incorporation and Cell Viability Assays for Cells Treated with Inhibitors—Cells were cultured with or without CSF-1 (360 ng/ml) in α-minimal essential medium supplemented with 10% newborn calf serum (Invitrogen). Cells with CSF-1 were preincubated in medium containing DMSO or SU6656 (2 μM), PD98058 (50 μM), LY294002 (10 μM), JNK inhibitor (10 μM), p38 MAPK inhibitor (5 μM), PLX3397 (15 μM), PLX5622 (15 μM), or their combinations for 4 h followed by an additional 14-h incubation in medium supplemented with 20 mM EdU. Incubation of cycling Mφ.WT macrophages with EdU for 14 h yielded maximum incorporation in the absence of inhibitors. Detection of EdU incorporation was performed according to the manufacturer’s instructions (Invitrogen). Each experiment contained controls of cells cultured with DMSO (no EdU), cultured with CSF-1 alone, or cultured without CSF-1 (+ EdU). Equivalent numbers of harvested cells were separately saved for measurement of cell viability by 7-aminoactinomycin D exclusion (34).

Chemical Cross-linking of Cell Surface CSF-1R—CSF-1-starved cells were incubated with or without 360 ng/ml CSF-1 at 4 °C for 2 h, washed three times with ice-cold PBS containing 25 mM HEPES, pH 7.0, and incubated with 1 mM BS3 in the same buffer for 8 min at 4 °C. The cross-linking reactions were stopped by washing twice with PBS and incubating with PBS containing 10 mM ammonium acetate for 2 min at 4 °C. After washing three times with ice-cold PBS, the cells were incubated with the goat anti-whole CSF-1R anti-serum for 15 min. The unbound antibodies were removed by centrifugation and four washes with ice-cold PBS. Cell lysate preparation and cell surface CSF-1R immunoprecipitation were performed as described (35).

CSF-1R Turnover Experiments—Cells were cultured in the presence and absence of CSF-1 (16 h), pulse-labeled (20 min) in Met/Cys-deficient Dulbecco’s modified Eagle’s medium containing 0.2 mCi/ml Trans35S-LABEL™ No-Thaw (1049 Ci/mmol), and chased in 100-fold excess of unlabeled Met and Cys as described (36). Alternatively, the cells were cultured for 16 h without CSF-1, cooled (4 °C for 15 min), washed (five times, cold PBS), and incubated in 1 mg/ml EZ-Link Sulfo-NHS-Biotin in PBS (30 min, 4 °C with gentle, continuous rocking) prior to replacement of the medium with 50 mM glycine in PBS, pH 7.0, at 4 °C followed by a rapid wash with CSF-1-deficient culture medium at 37 °C. In either case, the labeled cells were incubated with or without CSF-1 (360 ng/ml) for various times at 37 °C, and cell lysis, CSF-1R immunoprecipitation, SDS-PAGE, blotting, autoradiography, and staining (anti-CSF-1R and/or NeutrAvidin) were performed as described (31, 32).

RESULTS

Proliferation and Differentiation of Macrophages Expressing YEF CSF-1Rs with Single Tyrosine Add Backs—YEF CSF-1Rs, in which all eight tyrosines known to be phosphorylated in the...
activated CSF-1R are mutated to phenylalanine, were previously shown to lack in vitro kinase activity and CSF-1-stimulated tyrosine phosphorylation in vivo (20). MacCsf1 −/− (M −/−) macrophages expressing this receptor mutant (M −/−. YEF macrophages) were also unable to support CSF-1-stimulated survival, proliferation, or differentiation (20). To examine the sufficiency of each tyrosine to individually restore these activities, we infected M −/− macrophages with retroviruses encoding CSF-1Rs in which each tyrosine was added back to the YEF backbone (supplemental Fig. S1A). Following selection of macrophage lines expressing equivalent levels of cell surface receptor (supplemental Fig. S1B), the ability of cells of each CSF-1R single add-back mutant cell line to proliferate in the presence of CSF-1 was examined (supplemental Fig. S1C). When compared with M −/−.WT cells (doubling time, ~21 h), M −/−.YE.F559AB and M −/−.YE.F807AB cells (doubling times of ~30 and ~27 h, respectively) exhibited significant proliferation, and M −/−.YE.F697AB and M −/−.YE.Y721AB cells (doubling times of ~45 and ~65 h, respectively) exhibited a lower degree of proliferation, whereas other add backs had no significant effect (supplemental Fig. S1C). All single add-back lines cultured in the presence of CSF-1 exhibited less than 40% of the differentiation (Mac1 expression) of M −/−.WT macrophages (data not shown).

M −/−.YE.807AB Macrophage Proliferation Is Constitutive, whereas M −/−.YE.F,559,807AB Macrophages Exhibit Wild Type, CSF-1-dependent Proliferation—As macrophage proliferation was most severely affected by Tyr-559 and Tyr-807 mutations (20, 22) and most effectively rescued by their add backs (supplemental Fig. S1C), we further examined the contribution of Tyr-559 and Tyr-807 to CSF-1R activation and macrophage proliferation, survival, and differentiation in single add-back and double add-back (M −/−.YE.F559,807AB) macrophages, in the presence and absence of CSF-1 (Fig. 1, A and B). Surprisingly, M −/−.YE.F807AB macrophages proliferated at wild type rates independently of CSF-1 (Fig. 1A). In contrast, M −/−.YE.F559AB cells exhibited a low proliferative response without CSF-1 that was enhanced with CSF-1. Interestingly, M −/−.YE.F559,807AB macrophages proliferated like cells expressing the wild type receptor, exhibiting a normal response to CSF-1 and dying in its absence (Fig. 1B). When compared with M −/−.vector or M −/−.YE cells, which die in the presence of CSF-1 (20) (Fig. 1D), all three add-back lines survived well at 0.6 ng/ml CSF-1, which supports survival without proliferation (37) (Fig. 1D). However, all three exhibited a reduced ability to differentiate in response to CSF-1 (Fig. 1E), implying that differentiation requires other phosphotyrosine-based signaling pathways. Thus the single add back of Tyr-807 confers constitutive proliferation, which, in the absence of CSF-1, is blocked by the add back of Tyr-559, and this block is removed on stimulation of Y559,807AB macrophages with CSF-1.

In Vitro, Y559AB, Y807AB and Y559,Y807AB Receptors Possess Kinase Activity and Autophosphorylate, but Restoration of WT Levels Requires Tyr-544 in Addition—In vitro, the purified CSF-1R exhibits both autophosphorylation and kinase activity on exogenous substrates in the absence of CSF-1, with slight enhancement in the presence of CSF-1 (38). It was previously shown that the in vitro kinase activity of Y559F receptors was reduced when compared with wild type, but that Y807F kinase activity was not (20). The in vitro kinase activity and autophosphorylation of add-back CSF-1Rs were therefore examined in CSF-1R immunoprecipitates in the absence of CSF-1, using the optimum conditions described by Yu et al. (20) (Fig. 2). YEF or control kinase-dead K614A CSF-1Rs lacked kinase activity, and autophosphorylation was absent or barely detectable (Fig. 2). Each of the add-back CSF-1Rs examined had significant in vitro tyrosine kinase activity. There was a graded increase in their kinase activities (Y559AB, ~20% WT; Y807AB, ~40% WT; Y559,807AB, ~70% WT; Y544,559,807AB, ~100% WT) (Fig. 2A). However, receptors from both Y559AB and Y559,807AB macrophages exhibited low Tyr-559 phosphorylation (Fig. 2B). We have previously shown that the Y544F mutation, part of the YEF background, compromises in vitro receptor kinase activity and autophosphorylation, including phosphorylation of Tyr-559 (20). We therefore added back Tyr-544 to the Y559,807AB background (supplemental Fig. S1, A and B). For Y544,559,807AB receptors, both the in vitro kinase activity (Fig. 2A) and the in vitro Tyr-559 phosphorylation (Fig. 2B) were restored to the WT range, and the proliferative response of M −/−.YE.F544,559,807AB macrophages was indistinguishable from the proliferative response of M −/−.WT macrophages (Fig. 1C). In contrast, their differentiation response was not fully restored (Fig. 1E). Thus add back of Tyr-544, Tyr-559, and Tyr-807 is minimally required for restoration of full in vitro CSF-1R kinase activity and autophosphorylation of Tyr-559 (the first tyrosine to be phosphorylated in the response (23)), as well as for full restoration of the proliferative response. Despite their possession of at least 40% of WT kinase activity, Y807AB and Y559,807AB receptors exhibited
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minimal in vitro Tyr-807 autophosphorylation (Fig. 2C). However, with the return of kinase activity to WT levels in Y544,559,807AB receptors, a significant increase in the phosphorylation of both Tyr-807 and Tyr-559 was observed.

Y807AB Macrophages Fail to Exhibit Receptor Tyrosine Phosphorylation in Vivo—We next determined tyrosine phosphorylation of the add-back receptors in the context of the macrophage (Fig. 3). YEF.Y559AB receptors (Fig. 3B) exhibited tyrosine phosphorylation of Tyr-559 with kinetics comparable with those of WT cells (Fig. 3A). In contrast, YEF.Y807AB receptors failed to exhibit detectable tyrosine phosphorylation (Fig. 3C). In addition, when compared with WT receptors, the phosphorylation of Tyr-559 in YEF.Y559AB and YEF.Y559,807AB receptors was also strongly suppressed (Fig. 3, A, B, and D). However, with the further addition of Tyr-544, in YEF.Y544,559,807AB receptors, the phosphorylation of Tyr-559 approximated the level of Tyr-559 phosphorylation in WT cells, and phosphorylation of Tyr-807 was evident (the level of Tyr-807 phosphorylation relative to WT cannot be determined as the sole available antibody that detects phospho-Tyr-807 (anti-human CSF-1R phospho-Tyr-809) also detects pTyr-697, which is strongly phosphorylated in the WT receptor (39) [data not shown]). To further enhance detection of CSF-1R tyrosine phosphorylation, the macromolecules were stimulated under optimal conditions, at 4 °C and in the presence of pervanadate, to inhibit the action of protein tyrosine phosphatases.

Even under these conditions, we failed to detect significant tyrosine phosphorylation in Y807AB macrophages. These results also indicate that CSF-1 responsiveness, reflected in no (M−/−.Y559,807AB) or slow (M−/−.Y559AB) proliferation in the absence of CSF-1, is correlated with Tyr-559 phosphorylation in the proliferative response to CSF-1, consistent with the role of phosphorylation of this JMD tyrosine in the relief of autoinhibition (20–22).

Cell Surface Y807AB Receptors Exhibit Significant Ligand-independent Turnover—Although WT receptors were dramatically down-regulated in response to CSF-1 (Fig. 4A), Y807AB receptor levels (Fig. 3C, lower panel) or cell surface expression (Fig. 4A) were unchanged. To discriminate between a steady-state level of Y807AB receptor with a high turnover rate versus stable expression with a low turnover rate, we compared Y807AB turnover with the turnover of YEF.Y559,807AB or WT receptors using two approaches. In one approach, cells were surface-labeled with biotin at 4 °C and then rapidly warmed to 37 °C and incubated for different times ± CSF-1, prior to analysis of the biotin-labeled receptors (Fig. 4B). For M−/−.Y559,807AB macrophages, CSF-1 caused a rapid depletion of cell surface-labeled receptors (t½ = 5 min) when compared with a barely perceptible decrease in the absence of CSF-1 (t½ ≥ 60 min). In contrast, in YEF.Y807AB macrophages, CSF-1 had no effect on the disappearance of biotin-labeled cell surface receptors, which disappeared at an intermediate rate (t½ = 60 min).

In the second approach, CSF-1-starved M−/−.YEF.807AB MAC or M−/−.WT macrophages were labeled with 35S]Met/Cys for 20 min at 37 °C and then chased (90 min) in excess unlabeled Met/Cys. During this chase period, most of the labeled receptor
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Thus in the presence or absence of CSF-1, Y807AB receptors are highly expressed at the cell surface, but their rate of turnover (t_1/2 ~ 60 min) is significantly faster than the turnover of WT or Y559,807AB receptors in the absence of CSF-1. The constant turnover of kinase-active Y807AB receptors renders the detection of downstream signaling difficult (see below and Fig. 7). Add back of Tyr-559 restores stable, cell surface expression of Y807AB receptors in the absence of CSF-1 as well as the rapid CSF-1-induced turnover, both characteristics of the WT receptor.

\[ M/-.YEY,807AB \]

Macroproliferation Is Dependent on Receptor Kinase Activity, but Independent of CSF-1 Dimerization—The Y807AB receptor possesses detectable in vitro tyrosine kinase activity (Fig. 2A). To test the requirement of CSF-1R kinase for M/-.YEY,807AB proliferation, we introduced the K614A kinase-dead mutation onto the Y807AB background. M/-.YEY,807AB,K614A macrophages expressing this receptor failed to proliferate (Fig. 5, A and B), demonstrating that the constitutive cell proliferation of Y807AB macrophages was mediated via receptor kinase activity. As dimerization is required for tyrosine phosphorylation and activation of the WT CSF-1R (40, 41), we investigated whether M/-.YEY,807AB macrophage proliferation in the absence of CSF-1 was associated with CSF-1-independent receptor dimerization. Chemical cross-linking experiments showed that CSF-1 induced dimerization of both WT and Y807AB receptors, but neither were dimerized in the unliganded state (Fig. 5C). As we failed to detect dimerization of Y807AB receptors in the absence of CSF-1, although they are highly expressed on the cell surface, these results suggest that the Y807AB kinase activity required for proliferation is not dependent on cell surface receptor dimerization.

\[ M/-.YEY,807AB \]

Proliferation Signaling Pathways Are A Subset of Those Regulating Proliferation in M/-.YEY,544,559,807AB and M/-.WT Macrophages—To determine whether the Y807AB signaling pathways for proliferation are utilized by the WT receptor, we compared the effects of inhibitors of downstream kinases on M/-.YEY,807AB and M/-.WT proliferation, as assessed by Edu incorporation. We also examined M/-.YEY,544,559,807AB macrophages because the add back of tyrosines 544, 559, and 807 is the minimum add back restoring WT levels of in vitro tyrosine kinase activity (Fig. 2A) and the in vivo proliferative response to CSF-1 (Fig. 1C). The cell proliferation of all lines was inhibited, as expected, by the CSF-1R tyrosine kinase inhibitors, PLX3397 and PLX5622, and unaffected by JNK or p38 MAPK inhibitors (Fig. 6). M/-.YEY,807AB macrophages were strongly inhibited by the MEK inhibitor, PD98058, and the PI3K inhibitor, LY294002, but unaffected by the Src family kinase (SKF) inhibitor, SU6656. In contrast, the proliferation of M/-.WT and M/-.YEY,544,559,807AB macrophages was inhibited by the SFK inhibitor, as well as by the MEK and PI3K inhibitors. As expected, their proliferation was also inhibited by removal of CSF-1. With the exception of the CSF-1R kinase inhibitors, none of the inhibitor treatments had a major effect on cell viability (supplemental Fig. S3), and in a single experiment, combinations of inhibitors had additive effects (supplemental Fig. S4).

We therefore examined cellular tyrosine phosphorylation and the activation of Akt, ERK1/2, p38, and SFK in these cells with and without CSF-1 stimulation. Protein tyrosine phos-

is converted to the mature ~165-kDa form (36). The cells were then incubated ± CSF-1 for different times prior to immunoprecipitation and analysis of the labeled receptors (Fig. 4C). Similar to the biotinylation results, the turnover of the 35S-labeled ~165-kDa CSF-1R in M/-.WT macrophages mimicked those for the turnover of biotin-labeled receptors in M/-.YEY,559,807AB macrophages (+ CSF-1, t_1/2 = 5 min; -CSF-1, t_1/2 > 60 min), whereas the receptor turnover rate ± CSF-1 in M/-.YEY,807AB macrophages was intermediate between values ± CSF-1 for the WT or Y559,807AB receptors.
The phosphorylation of Y807AB cells was at basal levels and not affected by CSF-1 stimulation. They also failed to exhibit detectable constitutive or induced phosphorylation of Akt, ERK1/2, or p38 (Fig. 7). Given the high turnover rate of the Y807AB receptors, their constitutively active state, and the fact that these responses are transient and already down-regulated in similarly proliferating macrophages expressing the WT receptor, these results are not unexpected. In contrast, M/H11002/H11002/YEF.Y559AB, M/H11002/H11002/YEF.Y559,807AB, and M/H11002/H11002/YEF.Y544,559,807AB macrophages exhibited wild type levels of tyrosine phosphorylation of non-CSF-1R proteins (<130-kDa), and CSF-1-induced phosphorylation of all three downstream kinases was induced to WT levels in M/H11002/YEF.Y807AB macrophages. These three lines each contain Tyr-559, necessary and sufficient for binding and activation of SFK (21, 23, 24). Semiquantitative RT-PCR revealed that the mRNAs for six SFKs are expressed in both bone marrow-derived macrophages and M/H11002/YEF.Y807AB macrophages with Hck, Fgr, Lyn, and Fyn being most highly expressed (supplemental Fig. S5). Experiments by our group and others (42, 43) indicate that Hck, Fgr, and Lyn alone or together have no effect or inhibit (43) CSF-1-induced macrophage proliferation. In macrophages of the three Tyr-559-containing lines, but not M/H11002/YEF.Y807AB macrophages, CSF-1 activated Hck (pTyr-411), as reported previously (42), and SFK activation loop tyrosine phosphorylation was detected by anti-Src (Tyr-416) antibodies (supplemental Fig. S6). In contrast, macrophages of all lines, including M/H11002/YEF.Y807AB, exhibited constitutive phosphorylation of the inhibitory SFK sites detected by anti-Src (pY527) antibodies. From these experiments, it is not clear which SFK member contributes to the CSF-1 proliferative response indicated by the SU6656 inhibition. However, CSF-1-induced activation of SFK was observed in the lines that were inhibited by SU6656 and not in M/H11002/YEF.Y807AB macrophages.

Together, these data suggest that a significant component of the constitutive proliferative activity of YEF.Y807AB receptors is mediated by PI 3-kinase and ERK1/2 pathways that are a subset of those utilized by the WT and YEF.Y544,559,807AB receptors. They also demonstrate that the phospho-Tyr-559 site, necessary and sufficient for binding and activation of SFK (21, 23, 24), also contributes and additionally plays a significant role in regulating the tyrosine phosphorylation of nonreceptor macrophage proteins.

**DISCUSSION**

We have studied the roles of the JMD Tyr-559 and the AL Tyr-807, two CSF-1R tyrosines that play a critical role in regulating both CSF-1R activity and receptor-mediated macrophage proliferation. When added back to the YEF background, the AL Tyr-807 conferred proliferation rates close to those of M/H11002/WT macrophages. Thus Tyr-807 was established as the main driver of proliferation. In vitro, Y807AB receptors pos-

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**FIGURE 5.** In absence of CSF-1, constitutively active cell surface Y807AB receptors are not dimerized, but their activity is receptor kinase-dependent. A and B, the CSF-1R K614A ATP-binding site mutation blocks survival and proliferation in the context of both the WT and the Y807AB receptors. A, growth curves. Filled symbols, + CSF-1; open symbols, − CSF-1; means ± S.D., n = 3. B, cells were treated with or without CSF-1 at 4 °C for 2 h followed by two washes with ice-cold PBS, treatment with and without BS3 cross-linker for 8 min at 4 °C, cell lysis and analysis of the CSF-1Rs by immunoprecipitation (IP), SDS-PAGE, and Western blotting (WB). MuRD, multiquitinated receptor dimer (Tyr-559-dependent); RD, receptor dimer.
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FIGURE 6. M−/−.YEF.Y807AB macrophage proliferation signaling pathways are a subset of those regulating proliferation in YEF. Y544,559,807AB and WT macrophages. Macrophages were incubated in medium containing vehicle (DMSO) or inhibitor for 4 h followed by an additional 14 h incubation in medium supplemented with 20 mM EdU prior to collection and detection of EdU incorporation by flow cytometry (supplemental Fig. S2). The dotted lines indicate the baseline inhibitory activity of control cells that received EdU alone. Means ± S.D. of three separate experiments. *p < 0.05, Student’s t test. Note: These PLX inhibitors are not as active against the active conformation of the kinase, created in the Y807AB, as they are against the conformation of the wild type kinase. p38 MAPK inhib, p38 MAPK inhibitor.

**FIGURE 7.** Failure to detect activated ERK1/2 or Akt or above background levels of activated p38 MAPK in Y807AB macrophages and reconstitution of cellular tyrosine phosphorylation, ERK1/2, Akt, and p38 MAPK responses in M−/−.YEF.Y544,559,807AB macrophages. Cell lysis, SDS-PAGE, and Western blotting were carried out as described under “Experimental Procedures.” WB, Western blot; pTyr, phosphotyrosine; pAKT, phospho-Akt; pERK1/2, phospho-ERK1/2; pp38-MAPK, phospho-p38 MAPK.

...detected (Fig. 3C), the proliferative response of Y807AB macrophages required CSF-1R kinase activity (Figs. 5, A and B, and 6). Thus despite the dependence of proliferation on both CSF-1R kinase activity (Fig. 6) and AL Tyr-807 (20), Tyr-807 phosphorylation was not detected. This lack of a requirement for AL tyrosine phosphorylation contrasts with the early autophosphorylation of the AL Tyr-654 of the fibroblast growth factor receptor 1 kinase domain, which leads to a 50–100-fold increase in catalytic activity (44, 45), but resembles the closely related CSF-1R family member, c-Kit, in which there is very late tyrosine phosphorylation of the AL tyrosine (Tyr-823), indicating that Tyr-823 phosphorylation is also not required for activation (46). The results of a previous study using chimeric CSF-1Rs (22), in which it was suggested that Tyr-697 is required with Tyr-559 to regulate autophosphorylation of Tyr-807 and that Tyr-807 phosphorylation was restored to WT levels in YEF.Y544,559,697,807AB macrophages, should be interpreted differently as the anti-human CSF-1R phospho-Tyr-809 antibody used to detect phospho-Tyr-807 in those studies strongly cross-reacts with phospho-Tyr-697 (data not shown). However, as in the present study, no significant Tyr-807 phosphorylation was observed in Tyr-807AB or Tyr-559,807AB cells. CSF-1 induced dimerization of both WT and Y807AB cell surface receptors, indicating that the cell surface Y807AB receptors were available for dimerization. As the cell surface Y807AB receptor turnover studies indicated that significant Y807AB signaling occurs from the cell surface, these cross-linking studies suggest that the signaling from the Y807AB receptor in the absence of CSF-1 does not involve ligand-independent receptor dimerization. Relevant to the ability of Y807AB to signal proliferation autonomously in this fashion, both the fusion protein from a t(3;5)(p21;q33) translocation in acute megakaryoblastic leukemia and the region of the CSF-1R involved, which lacks the JMD and dimerization domains, induce a myeloid proliferative disease with features of megakaryoblastic leukemia in a murine transplant model (47).

Based on their crystal structure of the autoinhibited human CSF-1R kinase domain, Walter et al. (17) proposed that the unphosphorylated Tyr-809 (human equivalent of Tyr-807) points directly into the active site, acting as a pseudosubstrate surrounded by hydrophobic residues (Pro-818, Ile-803), with its hydroxyl group hydrogen bonding to the PDGFR family conserved catalytic residues, Asp-7780D2 and Arg-801NH2, and that together, these interactions contribute to stabilize an inhibited conformation of the activation loop. Our findings of the inactivity of YEF (Phe-807) and the activity of YEF.Y807AB in the absence of Tyr-807 phosphorylation support the importance of the hydrogen bonding of Tyr-807 in maintaining the structure of the active site. However, although the add back of Tyr-807 to YEF restored ~40% of the WT kinase activity and proliferation without CSF-1 almost to WT levels (Fig. 2A), the kinase activity of Y807F was similar to the kinase activity of the WT receptor (20), suggesting that other tyrosines besides Tyr-807 are involved in activation. Indeed, add back of Tyr-559 to YEF also restored ~20% of WT kinase activity (Fig. 2A), and some proliferation in the absence of CSF-1 (Fig. 1A) and Y559F had ~50% of WT kinase activity (20). Although single add backs of either Tyr-697 or Tyr-721 led to proliferation in the presence of...
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CSF-1, their proliferation rates were significantly lower than observed for add back of Tyr-559 (supplemental Fig. S1A), and they were not further examined. Studies with chimeric CSF-1R mutants overexpressed in primary bone marrow-derived macrophages have also shown that tyrosines 559, 807, 697, and 721 positively contribute to the proliferative response. However, no effect was observed when individual tyrosines were added back alone, and the overall reconstitution of proliferation appeared to be less with Y544,559,807AB cells (22). This difference may reflect a differential loss of proliferative capacity over the time required for preparation of the transduced macrophages (11 days), an effect of the constitutively activated Y807AB receptor on the maturation of the cells, and/or effects of the endogenous CSF-1R.

Tyr-559 is the first CSF-1R tyrosine to be phosphorylated in the response to CSF-1 (23). Consistent with this, Y559AB possessed in vitro kinase activity and detectable Tyr-559 phosphorylation (Fig. 2, A and B), and in vivo, Tyr-559 was phosphorylated with WT kinetics in YEF.Y559AB macrophages (Fig. 3). Tyr-559 alone also positively regulates proliferation, and proliferation was enhanced by CSF-1 due to phospho-Tyr-559-mediated activation of the SFK pathway (23) (Figs. 6 and 7). However, in the context of Y807AB and the absence of CSF-1 (M−/−.YEF.Y559,807AB macrophages), Tyr-559 completely inhibits Y807AB-mediated proliferation, reflecting its strong autoinhibitory role. The addition of CSF-1 to these cells leads to Tyr-559 phosphorylation and complete release of this inhibition (Fig. 3B) with barely detectable Tyr-807 tyrosine phosphorylation (Fig. 3D).

Earlier studies have pointed to roles of the ERK1/2, PI3K, and SFK pathways in the regulation of macrophage proliferation (22, 48, 49), and our results also indicate that multiple pathways are involved. Although no effector recruitment has been reported for Tyr-807, the inhibitor experiments indicate a major (Fig. 6) and additive (supplemental Fig. S4) involvement of the PI3K and ERK1/2 pathways in Y807AB-regulated macrophage proliferation. As we could not demonstrate induction of these downstream signaling pathways in M−/−.YEF.Y807AB macrophages incubated with CSF-1 (Fig. 7), we attempted to develop an alternate system in which to study Y807AB signaling, utilizing serum stimulation experiments in which the cells were serum-starved (0.1% serum), prior to stimulation with 10% serum. Lowering of serum concentrations lowered protein synthetic rates, which, because of the relatively high turnover of Y807AB receptors (Fig. 4, B and C), dramatically down-regulated their expression, and we failed to observe Y807AB, ERK1/2, or PI3K phosphorylation (data not shown). Because we used inhibitors of the upstream kinases in these experiments (Fig. 6), it is possible that downstream pathways other than the Akt and ERK1/2 are involved, especially in the case of Akt, where activation may depend on cell context (50). However, as these pathways cannot be detected in WT macrophages growing in the presence of CSF-1 and require a period of ligand starvation to elicit transient activation in response to acute CSF-1 stimulation, it is also possible that there was constant, low level of activation of Akt and ERK1/2 that we were unable to detect in the constitutively proliferating M−/−.YEF.Y807AB macrophages.

Together, the inhibitor and signaling studies reveal that Tyr-559 phosphorylation not only relieves repression of receptor tyrosine kinase activity, but also positively contributes by activating SFK and restoring the full wild type proliferative response in M−/−.YEF.544,559,807AB macrophages. Interestingly, Tyr-559 also contributes significantly to the overall macrophage protein tyrosine phosphorylation response (Fig. 7). The recently described phospho-Tyr-559/SFK/c-Cbl pathway that results in receptor ubiquitination and amplifies the receptor tyrosine phosphorylation (23) may contribute to these positive effects. However, Tyr-559 phosphorylation can also exert negative regulation by mediating ligand-induced CSF-1R-mediated endocytosis and degradation (20, 21, 23, 25).

JMD Tyr-544 phosphorylation has only been reported in the v-fms oncoprotein (11). However, the Y544F CSF-1R exhibits markedly reduced in vitro kinase activity and in vivo tyrosine phosphorylation, including Tyr-559 phosphorylation (20). In contrast to the YEF.559,807AB receptor, the YEF.Y544,559,807AB receptor was shown to possess WT levels of in vitro kinase activity (Fig. 2A) and Tyr-559 autophosphorylation (Fig. 2B), levels of CSF-1-stimulated Tyr-559 phosphorylation in macrophages approaching those of WT cells (Fig. 3), fully restored CSF-1-induced proliferation (Figs. 1C and 2B), and activation to WT levels of the three proliferation signaling pathways (Fig. 7) (23). Tyr-544 is highly conserved in the PDGFR family of receptor tyrosine kinases and located in the juxtamembrane buried region of the JMD. Its hydroxyl group forms hydrogen bonds with a key conserved residue regulating interlobe plasticity in the kinase domain (17). Thus irrespective of whether Tyr-544 is phosphorylated in the WT receptor or not, it plays an important role in permitting phospho-Tyr-559-mediated functions.

CSF-1R phosphotyrosine-initiated signaling pathways regulate macrophage functions other than survival and proliferation (1), and when compared with M−/−.WT macrophages, M−/−.YEF.Y544,559,807AB macrophages are clearly compromised in their differentiation (Fig. 1E) and chemotactic (51) responses. Furthermore, evidence points to important roles for the other 5 remaining tyrosines in the regulation of morphological responses, differentiation, and chemotaxis (20, 51). Thus the M−/−.YEF.Y544,559,807AB receptor, with its wild type kinase activity and proliferation response, is an appropriate backbone on which to add back these other tyrosines, either singly or multiply, to assess their necessity and sufficiency for the mediation of such responses.

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REFERENCES
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