Macrophage colony-stimulating factor differentially regulates low density lipoprotein and transferrin receptors

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Abstract **Endocytosis mediated by both LDL receptors (LDLRs) and transferrin receptors (TfRs) occurs in clathrin-coated pits and requires specific tyrosine-based internalization sequences located in the cytoplasmic domain of these receptors. Internalization of these receptors is mediated by endocytic proteins that interact with the internalization domains. We previously showed that macrophage colony-stimulating factor (M-CSF) rapidly increases LDLR-dependent uptake and metabolism of LDL. To study the mechanism by which M-CSF regulates LDL uptake, we compared the effect of M-CSF on the internalization of LDL and transferrin (Tf). Our results show that M-CSF substantially increased the rate of LDLR internalization without increasing LDLR localization on the cell surface. In contrast, M-CSF treatment of macrophages rapidly increased the localization of TfR to the cell surface but did not alter the relative rate of Tf internalization. Moreover, M-CSF regulated TfR and LDLR via the activation of distinct signaling pathways. Recruitment of TfR to the cell surface was attenuated by phosphatidylinositol 3-kinase inhibitors, whereas stimulated LDL uptake was inhibited by the serine/threonine phosphatase inhibitor okadaic acid. Taken together, our results indicate that M-CSF differentially regulates receptors that undergo endocytosis and that increased LDL uptake results from a selective increase in the rate of LDLR internalization.**—Du, L., and S. R. Post. **Macrophage colony-stimulating factor differentially regulates low density lipoprotein and transferrin receptors. *J. Lipid Res.* 2004.** 45: **1733–1740.**

Supplementary key words clathrin • endocytosis • lipoprotein • metabolism • signaling

Defects in LDL internalization result in hypercholesterolemia and significantly increased risk of atherosclerosis and heart disease. The best characterized defects are mutations in the low density lipoprotein receptor (LDLR) that result in decreased receptor internalization. Like other endocytic receptors, LDLR internalization is largely dependent on short stretches of amino acids within the cytoplasmic domain that serve as internalization signals. Several sequences that regulate receptor endocytosis have been identified, including *a*) tyrosine-containing motifs with the consensus of YXXΦ, where Φ represents a bulky hydrophobic residue [e.g., NPVY in the LDL receptor (1) and YTRF in the transferrin (Tf) receptor (2, 3)]; *b*) dileucine motifs [e.g., Glut4 transporter (4) and IgG Fc-receptor (5)]; *c*) clusters of acidic amino acids [e.g., furin receptor (6, 7)]; *d*) β-arrestin binding domains [e.g., G protein-coupled receptors (8)]; and *e*) phosphorylation sites [e.g., G protein-coupled receptors, tyrosine kinase receptors (7, 9–15)]. These endocytic motifs interact with specific adaptor proteins to couple the receptor with clathrin.

Interactions between the endocytic motif of the LDLR and clathrin adaptor proteins permit constitutive receptor internalization at a rate that is not altered by ligand binding (16–20). Thus, it has been generally assumed that the rate of ligand internalization mediated by LDLR is regulated only by modulating receptor expression. Mechanisms involved in the transcriptional regulation of LDLR expression have been well studied [reviewed in ref. (21)], and until recently, pathways involved in the posttranscriptional regulation of LDLR function have not been defined. Our recent data demonstrate that LDLR-mediated lipoprotein uptake by macrophages is stimulated by acute treatment with macrophage colony-stimulating factor (M-CSF) without altering LDLR expression (22). In contrast, M-CSF failed to stimulate lipoprotein metabolism in peritoneal macrophages isolated from $LDLR^{-/-}$ mice, demonstrating that enhanced uptake was mediated by the LDLR (22). This suggests that the rate of LDLR endocytosis is also regulated by intracellular signaling cascades.

Abbreviations: ARH, autosomal-recessive hypercholesterolemia; EGF, epidermal growth factor; LDLR, low density lipoprotein receptor; M-CSF, macrophage colony-stimulating factor; PI3-kinase, phosphatidylinositol 3-kinase; PP1 and PP2A, protein phosphatases 1 and 2A; Tf, transferrin; TfR, transferrin receptor.

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The cytoplasmic sequence of the transferrin receptor (TfR) contains a tyrosine-based internalization motif (YTRF) that, like the NPVY motif of the LDLR, interacts directly with the AP-2 clathrin adaptor protein. Similarly, TfRs are constitutively internalized in clathrin-coated pits and labeled Tf is often used as a marker for studying constitutive receptor endocytosis. In the current study, we compared the effect of M-CSF on the internalization of LDL and Tf by macrophages. These experiments were based on the hypothesis that M-CSF stimulates clathrin-coated pit internalization and that ligand internalization by both the TfR and the LDLR would be similarly enhanced. However, our findings indicate that M-CSF differentially regulates TfR and LDLR internalization by activation of distinct signaling pathways.

EXPERIMENTAL PROCEDURES

Chemicals

DMEM supplemented with L-glutamine, DMEM with 25 mM HEPES but without sodium bicarbonate, and heat-inactivated FBS were purchased from Gibco-BRL (Grand Island, NY). Penicillin, streptomycin, human holo-Tf (iron-saturated), and wortmannin were purchased from Sigma (St. Louis, MO). Okadaic acid was from Calbiochem (San Diego, CA). Recombinant murine M-CSF was purchased from R&D Systems (Minneapolis, MN) and was solubilized in PBS containing 0.1% BSA. Na[^{125}I] was obtained from Amersham (Piscataway, NJ). Chicken anti-mouse LDLR antibody was kindly provided by Dr. Alan Daugherty (University of Kentucky). Fluorescein-labeled goat anti-chicken secondary antibody was from Aves Labs, Inc. (Tigard, OR). Normal goat antiserum was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

J774A.1 cells, a murine macrophage cell line, were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in DMEM containing penicillin (10 U/ml), streptomycin (10 μg/ml), and 10% FBS.

Lipoprotein isolation and radioiodination

LDL (d = 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation of EDTA-anticoagulated plasma obtained from healthy normolipidemic volunteers. LDL was dialyzed against saline containing 1 mM EDTA, pH 7.4. Lipoprotein preparations were sterilized by passage through 0.22 μm filters and stored at 4°C. Lipoprotein samples were analyzed for protein content as described by Lowry et al. (23). LDL was radiolabeled using an indirect labeling method with Na[^{125}I] using IODO-GEN®-precoated tubes (Pierce Chemical Co., Rockford, IL) as described previously (22, 24).

[^{125}I]LDL association and binding to macrophages

To quantify cell-surface lipoprotein binding, J774 macrophages were cultured on 12-well plates in the presence of DMEM plus antibiotics and serum. When the cells were 80% confluent, the medium was changed to serum-free DMEM plus antibiotics, and the macrophages were cultured for an additional 10 min in the absence or presence of M-CSF (25 ng/ml). To assess [^{125}I]LDL binding to surface receptors, the cell medium was replaced with ice-cold DMEM supplemented with 0.5% BSA and 25 mM HEPES, pH 7.4, and cells were incubated for 0.5 h at 4°C. [^{125}I]LDL (10 μg/ml) was added, and the cells were incubated for an additional 2 h in the absence or presence of a 40-fold excess of unlabeled LDL. The cells were then washed three times with ice-cold PBS, and cellular protein was solubilized for 16 h in 0.5 ml of 0.1 N NaOH. Radioactivity in the extract was determined using a γ counter, and cell protein was assayed using the Bio-Rad protein assay. The amount of specific binding was calculated by subtracting the amount of [^{125}I]LDL bound in the presence of a 40-fold excess of unlabeled LDL from the total amount of [^{125}I]LDL bound.

Total lipoprotein association with cells was quantified as described previously (22, 24). Briefly, J774 macrophages were cultured as described above and then incubated in the absence or presence of M-CSF (25 ng/ml) at 25°C for 10 min. [^{125}I]LDL (10 μg/ml) was added, and the cells were incubated for an additional 2 h at 25°C in the absence or presence of a 40-fold excess of unlabeled LDL. Lipoprotein association was stopped by washing the cells three times with ice-cold PBS. The amounts of cell-associated radioactivity and cell protein were determined as described above.

Tf labeling

Iron-saturated Tf (100 μg in 500 μl of PBS) was radiolabeled by incubating (3 min, 25°C) with Na[^{125}I] (1.0 μCi) in IODO-GEN®-precoated tubes according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). The labeled Tf protein was applied to an Econo-Pac® 10DG column (Bio-Rad Laboratories, Hercules, CA), preequilibrated with PBS containing 1 mg/ml BSA, and eluted with PBS. The specific activity (cpm/ng protein) of [^{125}I]Tf in the eluate was calculated according to the determination of the protein concentration using the Bio-Rad protein assay, and the amount of label incorporated was assessed using a γ counter.

[^{125}I]Tf association and internalization

To examine Tf association, cells were preincubated with M-CSF at 25°C as indicated. Cells were then either incubated with [^{125}I]Tf (0.21 μg/ml) for 10 min at 25°C to determine total cell association (surface-bound plus internalized) or were cooled to 4°C and incubated with [^{125}I]Tf (0.21 μg/ml) for 2 h to assess surface binding. Nonspecific binding of [^{125}I]Tf was determined in the presence of a 100-fold excess of unlabeled Tf. Results are presented as nanograms of [^{125}I]Tf bound per milligram of cell protein.

To measure the rate of Tf internalization, J774 macrophages were untreated or treated with M-CSF (25 ng/ml) for 10 min at 25°C and then incubated at 4°C for 2 h with [^{125}I]Tf (0.21 μg/ml), and cells were washed to remove unbound ligand and warmed to 25°C to allow for ligand internalization. Incubations were terminated by washing cells with either PBS (total association) or with a glycine-NaCl solution, pH 3.0 (internal). Radioactivity and cell protein were determined as described above. The amount of surface-bound ligand was calculated by subtracting the counts associated with cells after incubation with glycine-NaCl from the total association of [^{125}I]Tf.

Flow cytometry

To measure the rate of LDLR internalization, J774 macrophages were incubated in serum-free DMEM for 2 h at 37°C. Cells were collected and resuspended in ice-cold DMEH plus 0.5% BSA. Cells were incubated with 5% normal goat antiserum for 15 min at 4°C, followed by incubation with chicken anti-mouse LDLR antibody (1:50) for 1 h at 4°C. Cells were then washed with ice-cold DMEM plus 0.5% BSA three times and treated with or without M-CSF (25 ng/ml) for 5 min at 4°C. Cells were incubated at 25°C for the indicated times and then washed three times with ice-cold PBS plus 0.5% BSA. Fluorescein-labeled

goat anti-chicken secondary antibody (1:100) was added, and cells were incubated at 4°C for 1 h. Cells were then washed with ice-cold PBS plus 0.5% BSA, and fluorescence associated with cells was determined by flow cytometry.

RESULTS

M-CSF selectively regulates ligand binding to cell surface receptors

We previously reported that M-CSF increased LDL association with macrophages (22). To determine whether M-CSF similarly affected Tf association, J774 macrophages were treated with M-CSF and then incubated with [^{125}I]Tf for different times. Initial experiments were conducted at 37°C; however, ligand association was too rapid to accurately assess the rate. Therefore, experiments were carried out at 25°C to slow the rate of association. As shown in **Fig. 1A**, M-CSF increased the rate that [^{125}I]Tf associated with cells without affecting the maximal amount of [^{125}I]Tf bound. Because measurements of total Tf association at 25°C include both surface and internalized ligand, we used an acid-wash protocol to remove surface-bound ligand, thereby allowing determination of internalized [^{125}I]Tf. Incubation of cells with M-CSF for 10 min resulted in a 2.3- ± 0.3-fold (n = 3) increase in the amount of [^{125}I]Tf bound to the surface of J774 macrophages (Fig. 1B). Increased surface binding was observed by 5 min but was similar to control levels by 40 min. The effect of M-CSF on cell surface TfR was directly examined using ligand binding assays conducted at 4°C to prevent receptor internalization. As shown in Fig. 1C, pretreating J774 macrophages with increasing concentrations of M-CSF for 10 min at 25°C substantially increased the subsequent binding of [^{125}I]Tf to cells incubated at 4°C. The effect of M-CSF on TfR localization displayed an EC_{50} of ~3 ng/ml and was maximal by 25 ng/ml, consistent with its effect on LDLR-mediated ligand association (24). To determine whether M-CSF enhanced Tf binding by increasing receptor affinity or increasing receptor number, we measured [^{125}I]Tf binding using increasing concentrations of ligand. As shown in Fig. 1D, [^{125}I]Tf binding at 4°C to cells that were previously treated with M-CSF displayed a similar affinity for [^{125}I]Tf as untreated cells (K_d = 0.75 vs.

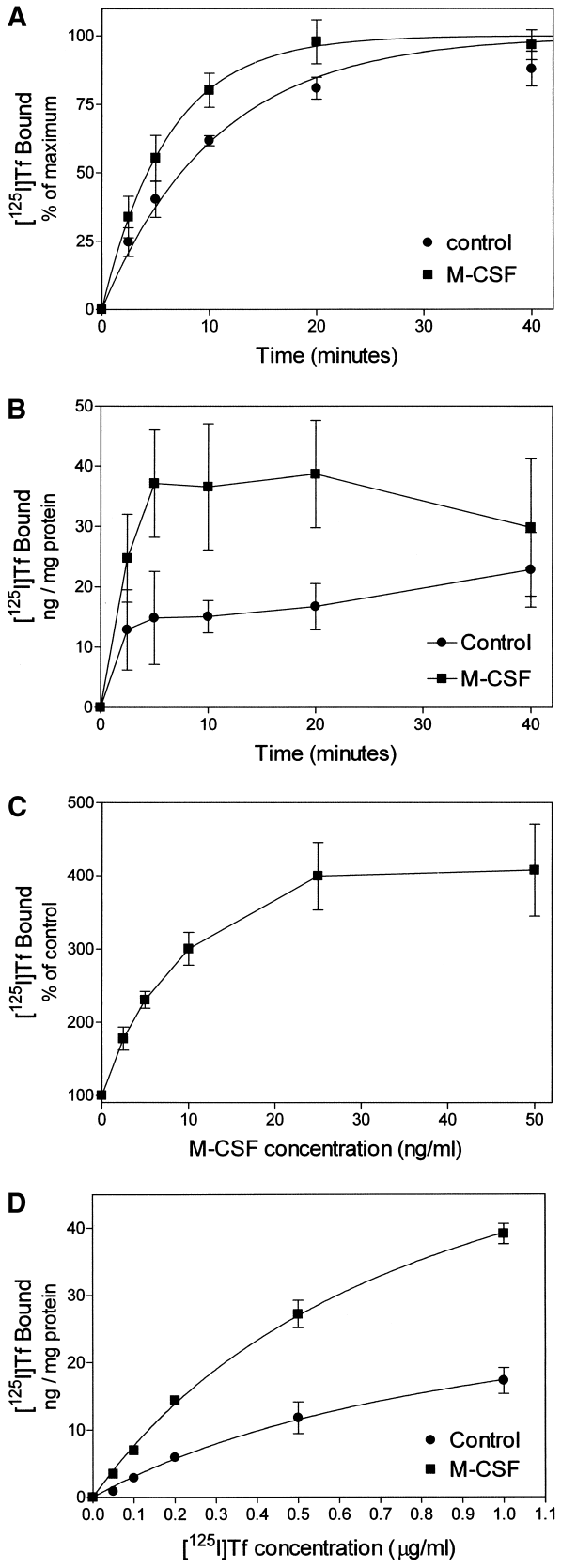

Fig. 1. Macrophage colony-stimulating factor (M-CSF) increases transferrin (Tf) binding to J774 macrophages. J774 macrophages were untreated or treated at 25°C with M-CSF for 10 min, then incubated with [^{125}I]Tf (0.21 μg/ml) for the indicated periods of time. The amount of specific binding was calculated by subtracting the amount of [^{125}I]Tf bound in the presence of a 100-fold excess of unlabeled Tf from the total amount of [^{125}I]Tf bound. A: Total cell association of [^{125}I]Tf was obtained after washing the cells with PBS. B: The internal amount of [^{125}I]Tf was obtained by washing the cells with a glycine-NaCl solution, pH 3.0. The amount of surface binding was calculated by subtracting the internal part from the total association of [^{125}I]Tf. C: J774 macrophages were untreated or treated at 25°C with increasing amounts of M-CSF for 10 min, then incubated with [^{125}I]Tf (0.21 μg/ml) for 10 min. The amount of surface binding of [^{125}I]Tf was determined as above. D: J774 macrophages were untreated or treated at 25°C with M-CSF (25 ng/ml) for 10 min, then incubated with increasing amounts of [^{125}I]Tf for 10 min. The amount of surface binding of [^{125}I]Tf was determined as above. Data represent the mean ± SD of at least three separate experiments.

0.60 ng/ml; n = 3, $P > 0.05$). However, cells previously treated with M-CSF bound substantially more [^{125}I]Tf, demonstrating that M-CSF treatment increased the total number of Tf binding sites. Together, these results indicate that M-CSF treatment of macrophages results in a rapid translocation of TfR to the cell surface.

Results from TfR binding assays indicated that M-CSF treatment promoted a rapid increase in the surface localization of TfR. To determine whether M-CSF induced a similar increase in cell surface localization of the LDLR, J774 macrophages were preincubated with M-CSF and subsequent ligand binding to cell surface receptors was determined at 4°C (**Fig. 2**). Consistent with results shown in Fig. 1, M-CSF pretreatment increased the subsequent binding of [^{125}I]Tf to cell surface receptors. In contrast, M-CSF pretreatment did not increase the binding of [^{125}I]LDL to cells, indicating that M-CSF differentially affects LDLR and TfR cellular localization.

M-CSF enhances the rate of LDLR internalization

An increase in LDLR-mediated ligand association without a parallel increase in binding to receptors on the cell surface suggests that M-CSF increases the rate of LDLR internalization. This possibility was directly assessed by examining the effect of M-CSF on the rate of LDLR antibody internalization. In the absence of M-CSF, LDLR antibody internalization was maximal by 10 min (**Fig. 3**), consistent with previously published rates for internalization via clathrin-coated pits (25, 26). In the presence of M-CSF, the rate of LDLR internalization was increased such that maximum antibody internalization was reached by 5 min. Importantly, M-CSF stimulated the rate but not the extent of antibody internalization, suggesting that M-CSF enhances LDLR cycling through the endocytic pathway.

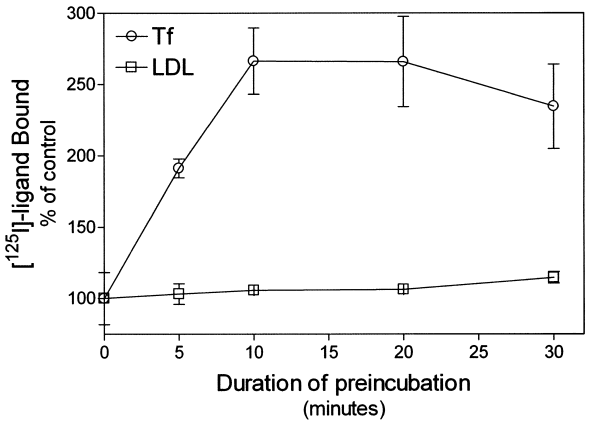

Fig. 2. M-CSF increases surface localization of Tf receptor but not low density lipoprotein receptor (LDLR). J774 macrophages were treated with M-CSF (25 ng/ml) at 25°C for the indicated periods of time. Cells were cooled and incubated with [^{125}I]Tf (0.21 μg/ml) or [^{125}I]LDL (10 μg/ml) for 2 h at 4°C to prevent ligand internalization. The amount of specific binding was calculated by subtracting the amount of ligand bound in the presence of an excess of unlabeled ligand from the amount of [^{125}I]ligand bound. Data shown are means ± ranges from at least two independent determinations.

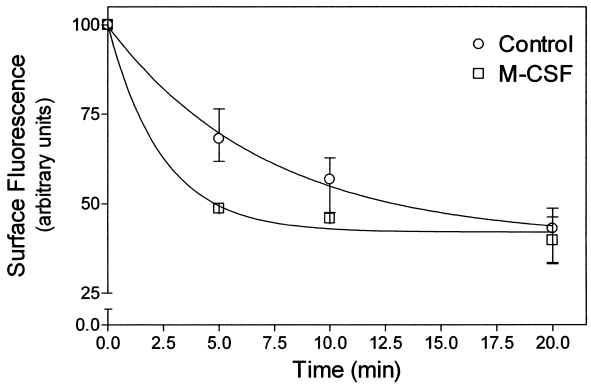

Fig. 3. M-CSF increases the rate of LDLR internalization. J774 macrophages were incubated with chicken anti-mouse LDLR antibody (1:50) for 1 h at 4°C and then washed with ice-cold DMEM plus 0.5% BSA. Cells were then treated with or without M-CSF (25 ng/ml) for 5 min at 4°C. After the cells were incubated in 25°C for the indicated times, cells were washed with ice-cold PBS plus 0.5% BSA. Fluorescein-labeled goat anti-chicken secondary antibody (1:100) was added, and cells were incubated at 4°C for 1 h. Cells were then washed with ice-cold PBS plus 0.5% BSA, and fluorescence associated with cells was determined by flow cytometry. Data shown are means ± ranges from at least two independent determinations.

M-CSF does not alter the rate of Tf internalization

Because M-CSF increases the rate of LDLR internalization and both the LDLR and TfR are endocytosed in clathrin-coated pits, we examined whether M-CSF pretreatment similarly enhanced the rate of TfR internalization. For this, cells were pretreated with M-CSF for 10 min at 25°C, cooled to 4°C, and then incubated with [^{125}I]Tf for 2 h. After removal of unbound ligand, the cells were incubated in the continued presence or absence of M-CSF for various times at 25°C to allow the surface-bound ligand to internalize, and the amount of total associated and internalized [^{125}I]Tf was determined. As shown in **Fig. 4A**, M-CSF treatment increased the total amount of Tf bound to cell surface receptors during the 4°C incubation and the total amount of [^{125}I]Tf associated with cells did not change during the incubation at 25°C. After incubation at 4°C, less than 15% of the total ligand was resistant to acid stripping, confirming that the bound ligand remained on the cell surface. However, greater than 70% of the cell-associated ligand was resistant to acid stripping after 20 min at 25°C, indicating that the ligand was internalized. Although M-CSF increased the amount of [^{125}I]Tf bound, the relative rate of ligand internalization was not altered in the presence of M-CSF (Fig. 4B). The differential effect of M-CSF on the internalization rates of the LDLR and TfR suggests that M-CSF does not induce a generalized increase in the rate of clathrin-coated pit internalization.

M-CSF regulation of TfR and LDLR involves different signaling pathways

In peritoneal macrophages, M-CSF increases LDLR-mediated lipoprotein uptake via activation of a pertussis toxin-sensitive signaling pathway (22). Therefore, we examined the role of pertussis toxin-sensitive signaling path-

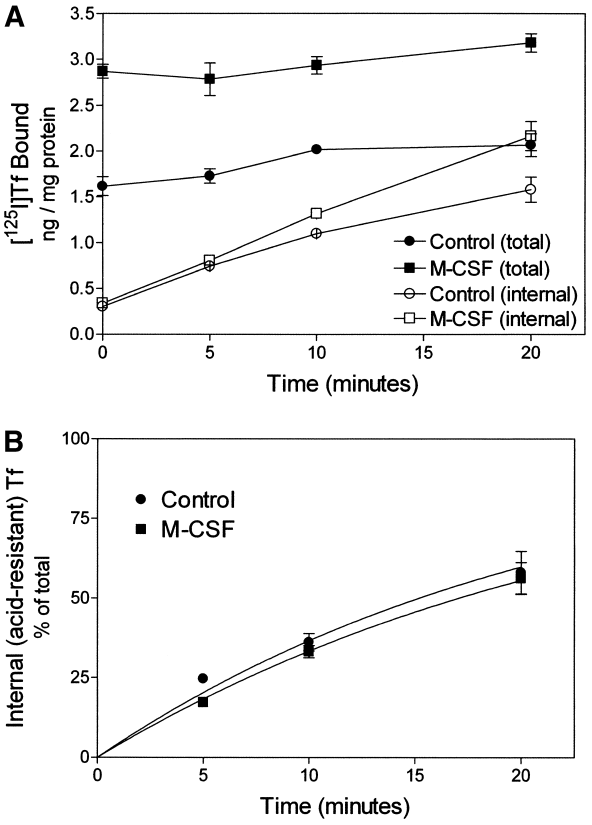

Fig. 4. M-CSF increases surface Tf binding but does not alter the rate of Tf internalization. A: J774 macrophages were untreated or treated with M-CSF (25 ng/ml) for 10 min at 25°C and then incubated at 4°C for 2 h with [^{125}I]Tf (0.21 μg/ml). Cells were washed to remove unbound ligand and warmed to 25°C to allow for ligand internalization. At the indicated times, incubations were terminated by washing cells with either PBS (total) or with a glycine-NaCl solution, pH 3.0 (internal). The amount of surface-bound ligand was calculated by subtracting the counts associated with cells after incubation with glycine-NaCl from the total association of [^{125}I]Tf. Specific binding was defined by incubation in the presence of an excess of unlabeled Tf. B: The relative rate of ligand internalization calculated by dividing the amount of internal ligand by the total amount of ligand associated with cells at each time point. Data represent the mean ± SD of at least three separate experiments.

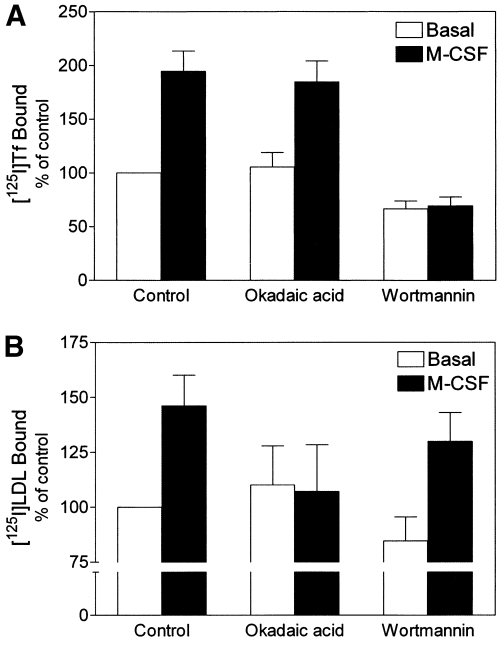

Fig. 5. M-CSF stimulates Tf binding and LDL uptake involves activation of different signaling pathways. As indicated, J774 macrophages were treated at 25°C with wortmannin (100 nM; 30 min) or okadaic acid (30 nM; 10 min), then treated with M-CSF (25 ng/ml) for 10 min. A: To determine Tf binding, cells were cooled and incubated with [^{125}I]Tf (0.21 μg/ml) for 2 h at 4°C. The amount of specific binding was calculated by subtracting the amount of ligand bound in the presence of a 100-fold excess of unlabeled Tf from the total amount of [^{125}I]ligand bound. B: For analysis of LDL association, cells were incubated with [^{125}I]LDL (10 μg/ml) for 2 h at 25°C. The amount of specific association was calculated by subtracting the amount of ligand bound in the presence of a 40-fold excess of unlabeled LDL from the amount of [^{125}I]ligand bound. Data shown are means ± ranges from at least two independent experiments.

ways in regulating TfR and LDLR in J774 macrophages. Unlike peritoneal macrophages, pertussis toxin treatment of J774 macrophages did not inhibit M-CSF-stimulated TfR surface localization or LDLR-mediated uptake (data not shown). Therefore, we examined the involvement of other M-CSF-stimulated signaling pathways in regulating the effects of M-CSF on TfR and LDLR. M-CSF binding to its receptor activates multiple signaling cascades, resulting in changes in protein and lipid phosphorylation (27). For example, M-CSF activates phosphatidylinositol 3-kinase (PI3-kinase), and PI3-kinase can regulate endocytosis and vesicular trafficking (28–31). We next examined whether specific inhibition of PI3-kinase affected the ability of M-CSF to stimulate uptake via the LDLR and TfR. As shown in **Fig. 5A**, pretreatment of cells with wortmannin (100 nM) completely inhibited M-CSF stimulation of [^{125}I]Tf binding to cell surface receptors. Similar results were obtained using LY294002 to inhibit PI3-kinase (data not shown). In contrast to its effect on [^{125}I]Tf binding, M-CSF-enhanced [^{125}I]LDL association was not inhibited by wortmannin (Fig. 5B). To determine whether changes in protein phosphorylation were involved in M-CSF-mediated regulation of TfR and LDLR, cells were treated with okadaic acid, an inhibitor of the serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A). As shown in Fig. 5, okadaic acid pretreatment did not affect the ability of M-CSF to regulate TfR surface localization but did inhibit the regulation of LDLR internalization. Together, these results demonstrate that the effect of M-CSF on LDLR and TfR is mediated by different intracellular signaling pathways.

DISCUSSION

Endocytosis of cell surface receptors is largely dependent on specific internalization motifs located within the receptor's cytoplasmic domains. For example, the cytoplasmic domains of both the LDLR and TfR contain tyrosine-based internalization signals: NPVY in the LDLR and

YTRF in the TfR (1–3). Because these receptors display extensive colocalization at the cell membrane and within intracellular vesicles (25), we hypothesized that M-CSF would similarly regulate LDL and Tf internalization. However, the current results indicate that TfR- and LDLR-mediated endocytosis are differentially regulated by M-CSF.

It was previously reported that M-CSF enhances LDLR-mediated lipoprotein degradation in macrophages (22). The finding that M-CSF increases the rate of LDLR internalization without increasing LDLR surface localization indicates that M-CSF increases LDL uptake by enhancing LDLR-mediated endocytosis. This effect does not result from a generalized increase in clathrin-coated pit-dependent endocytosis, as the rate of TfR internalization was not enhanced by M-CSF. Okadaic acid inhibited the effect of M-CSF on LDLR-mediated uptake, demonstrating an important role for serine/threonine phosphorylation. In contrast to its effect on LDLR, M-CSF increased TfR surface localization via the activation of a PI3-kinase-dependent pathway. Together, these data support the conclusion that M-CSF differentially regulates TfR- and LDLR-mediated endocytosis by activation of specific intracellular signaling pathways.

Based on current understanding of LDLR and TfR structure and regulation, there are several possible mechanisms that may explain the differential effects of M-CSF on these two endocytic receptors. One possibility is that the LDLR and TfR interact with different adaptor proteins that regulate receptor interaction with clathrin-coated pits (32–37). One such adaptor protein, AP-2, recruits endocytic receptors to coated pits by recognizing specific sorting signals present in the cytosolic tails of membrane proteins. In particular, the μ-subunit of the AP-2 complex interacts with the YXXΦ internalization motif initially identified in the TfR. However, the NPVY motif found in the LDLR interacts only weakly with the terminal domain of clathrin and with AP-2 (16), suggesting that the LDLR interacts with other adaptor proteins to couple to clathrin-coated pit-mediated endocytosis. This possibility is supported by the observation that depleting cells of the AP-2 adaptor protein with small-inhibitory RNA inhibited TfR internalization without decreasing endocytosis mediated by either LDL or epidermal growth factor (EGF) receptors (38). Additional support is derived from experiments that demonstrate a lack of competition between the endocytic signals of the TfR, LDLR, and EGF receptor when the different receptor types are overexpressed in cells (39). Together, these data provide compelling evidence that LDLR and TfR are able to interact with distinct adaptor proteins. Our results suggest that an interaction between the LDLR and one or more adaptor proteins may be regulated by M-CSF through a serine/threonine phosphorylation-dependent pathway.

In addition to AP-2, many other clathrin adaptor proteins that selectively recognize different internalization sequences have been described [reviewed in ref. (33)]. For example, Dab1 and Dab2 are two adaptor proteins that have been shown to interact with the NPVY motif of the LDLR and related proteins (40–43). Moreover, overexpressing a dominant-negative Dab2 construct inhibited LDLR- but not TfR-mediated endocytosis (41). Similar to Dab2, the autosomal-recessive hypercholesterolemia (ARH) protein was identified as a clathrin adaptor protein that regulates LDLR internalization in hepatocytes, lymphocytes, and macrophages (44–46). The severe hypercholesterolemia that occurs in individuals with mutations in this gene indicates a crucial role for ARH in regulating LDLR function. Recently, an interaction between the LDLR and β-arrestin2 was demonstrated (47). β-Arrestins are typically associated with regulating the internalization of G-protein-coupled receptors that have been phosphorylated by a G-protein receptor kinase. The interaction between LDLR and β-arrestin2 was physiologically important in that β-arrestin2$^{-/-}$ mice fed a high-fat diet had increased LDL cholesterol levels and cells isolated from these mice displayed substantially less LDLR endocytosis. Interestingly, a S833D mutant of the LDLR, which should mimic the endogenous phosphorylation of the receptor (48, 49), significantly increased the affinity of the receptor for β-arrestin2, suggesting that phosphorylation of LDLR enhances interaction with β-arrestin2. Overall, our results are consistent with a model whereby M-CSF-mediated phosphorylation of the LDLR and/or LDLR-selective adaptor proteins on serine/threonine specifically regulates LDLR endocytosis. The specific role of individual adaptor proteins in the M-CSF-dependent regulation of LDLR endocytosis is the focus of ongoing investigation.

An alternative explanation for the observed differential effects of M-CSF on LDLR and TfR trafficking is that M-CSF stimulates LDLR internalization via a pathway other than that involved in constitutive internalization (i.e., clathrin-coated pits). For example, LDL uptake by fluid-phase endocytosis (e.g., macropinocytosis) has been demonstrated in both pigeon and human macrophages (50, 51). Furthermore, EGF stimulates macropinocytosis and internalization of the EGF receptor via a clathrin-independent pathway (52), suggesting that enhancing macropinocytosis increases receptor-dependent endocytosis independent of clathrin-coated pits. Because M-CSF also stimulates macropinocytosis (53, 54), it is possible that M-CSF stimulates LDL uptake via a pathway distinct from clathrin-coated pit-mediated endocytosis.

Activation of intracellular signaling pathways is well known to regulate endocytosis and receptor trafficking. In particular, several studies support a role for PI3-kinase activation and phosphoinositide metabolites in vesicular trafficking [reviewed in refs. (55, 56)]. For example, the PI3-kinase inhibitors wortmannin and LY294002 have been shown to inhibit receptor-independent uptake by fluid-phase pinocytosis without affecting receptor-mediated endocytosis (53). In addition, insulin-induced recruitment of both Glut4 and TfR to the cell surface of 3T3-L1 adipocytes involves PI3-kinase activation (57, 58). In these studies, protein translocation to the cell surface was attributed to an increase in receptor translocation from an intracellular pool to the plasma membrane with no apparent effect on endocytosis. This is similar to our results demonstrating that in J774 macrophages PI3-

kinase activation enhances the localization of TfR, but not LDLR, to the cell surface without altering the rate of Tf internalization. Although less well studied, okadaic acid is also associated with regulating both fluid-phase and receptor-mediated endocytosis (59–61). For many receptors, internalization requires the phosphorylation of specific serine/threonine residues. The subsequent recycling of these receptors involves the dephosphorylation of the internalized receptor by a PP1/PP2A protein phosphatase (60–62). Inhibition of these phosphatases interrupts the endocytic process and inhibits receptor function. The okadaic acid sensitivity of M-CSF-stimulated LDL uptake suggests that a similar regulatory process may regulate LDLR trafficking.

In summary, M-CSF differentially regulates ligand endocytosis mediated by TfR and LDLR in macrophages. M-CSF stimulates a rapid, PI3-kinase-dependent translocation of TfR from an intracellular compartment to the cell surface without altering the relative rate at which these receptors are internalized. In contrast to its effect on TfR, M-CSF increases the rate of LDLR endocytosis without altering the cell surface localization of LDLR. Regulation of the LDLR is sensitive to changes in serine/threonine phosphorylation but is independent of PI3-kinase. Overall, our results suggest that LDLR-mediated lipoprotein endocytosis is selectively regulated by M-CSF-dependent signaling cascades.

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