THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 275, No. 46, Issue of November 17, pp. 35807–35813, 2000
Printed in U.S.A.

Macrophage Colony-stimulating Factor Rapidly Enhances β-Migrating Very Low Density Lipoprotein Metabolism in Macrophages through Activation of a $G_{i/o}$ Protein Signaling Pathway*

Received for publication, March 3, 2000, and in revised form, August 22, 2000
Published, JBC Papers in Press, August 29, 2000, DOI 10.1074/jbc.M001797200

Stewart C. Whitman‡§, Alan Daugherty‡, and Steven R. Post¶‖

From the ‡Division of Cardiovascular Medicine and ¶Department of Pharmacology, Atherosclerosis Research Group, Linda and Jack Gill Heart Institute, University of Kentucky, Lexington, Kentucky 40536-0284

Previous studies have examined lipoprotein metabolism by macrophages following prolonged exposure (>24 h) to macrophage colony-stimulating factor (M-CSF). Because M-CSF activates several signaling pathways that could rapidly affect lipoprotein metabolism, we examined whether acute exposure of macrophages to M-CSF alters the metabolism of either native or modified lipoproteins. Acute incubation of cultured J774 macrophages and resident mouse peritoneal macrophages with M-CSF markedly enhanced low density lipoproteins (LDL) and β-migrating very low density lipoproteins (β-VLDL) stimulated cholesteryl [^{3}H]oleate deposition. In parallel, M-CSF treatment increased the association and degradation of ^{125}I-labeled LDL or β-VLDL without altering the amount of lipoprotein bound to the cell surface. The increase in LDL and β-VLDL metabolism did not reflect a generalized effect on lipoprotein endocytosis and metabolism because M-CSF did not alter cholesterol deposition during incubation with acetylated LDL. Moreover, M-CSF did not augment β-VLDL cholesterol deposition in macrophages from LDL receptor (−/−) mice, indicating that the effect of M-CSF was mediated by the LDL receptor. Incubation of macrophages with pertussis toxin, a specific inhibitor of $G_{i/o}$ protein signaling, had no effect on cholesterol deposition during incubation with β-VLDL alone, but completely blocked the augmented response promoted by M-CSF. In addition, incubation of macrophages with the direct $G_{i/o}$ protein activator, mastoparan, mimicked the effect of M-CSF by enhancing cholesterol deposition in cells incubated with β-VLDL, but not acetylated LDL. In summary, M-CSF rapidly enhances LDL receptor-mediated metabolism of native lipoproteins by macrophages through activation of a $G_{i/o}$ protein signaling pathway. Together, these findings describe a novel pathway for regulating lipoprotein metabolism.

Macrophage colony-stimulating factor (M-CSF),[1] also known as colony-stimulating factor-1, is a homodimeric glycoprotein synthesized by a variety of cell types including monocytes (1), macrophages (1), endothelial cells (2), fibroblasts (3), and lymphocytes (4). M-CSF plays an important role in the proliferation, differentiation, and survival of monocytes (5–8) and in regulating macrophage function (6). M-CSF acts on monocytes and macrophages by binding with high affinity to the M-CSF receptor, a member of the protein-tyrosine kinase receptor subfamily encoded by the c-*fms* protooncogene (9, 10).

Following M-CSF binding, the M-CSF receptor autophosphorylates and mediates tyrosine phosphorylation of other substrate proteins thereby initiating a number of well defined signaling cascades, including activation of phosphatidylinositol 3-kinase (PI 3-kinase), the non-receptor Src family of tyrosine kinases, and pertussis toxin-sensitive guanosine triphosphate-binding proteins ($G_{i/o}$ proteins) (11). Previously published findings have shown that PI 3-kinase activation enhances receptor-mediated endocytosis via clathrin-coated pits (12), that Src family kinase activation is often associated with cytoskeletal changes (13), and that activation of G protein-coupled signaling pathways modulates endocytosis of the low density lipoprotein (LDL) receptor-related protein, an LDL receptor family member that can bind and internalize cholesterol ester-rich β-migrating very low density lipoproteins (β-VLDL) (14). In addition, we recently demonstrated a novel regulatory pathway in macrophages linking signaling through $G_{i/o}$ proteins and scavenger receptor class A (SR-A)-mediated uptake of acetylated low density lipoproteins (AcLDL) (15).

Atherosclerosis is thought to result from an alteration in lipoprotein metabolism precipitated by a chronic inflammatory process involving T-lymphocytes and macrophages within the vascular wall. Human and rabbit atherosclerotic lesions have been shown to contain mRNA and protein for both M-CSF (16, 17) and its receptor (18). M-CSF can directly affect multiple steps involved in atherogenesis including monocyte recruitment, macrophage survival and proliferation within the lesion, and removal of modified lipoproteins from the extracellular space of the vessel wall. Results from *in vivo* studies have suggested that M-CSF has both pro- and anti-atherogenic activities (19–22). Atherosclerotic studies using osteopetrotic (op/op) mice, which lack M-CSF due to a structural gene mutation,

* This work was supported, in part, by an atorvastatin research award from Pfizer and University of Kentucky Research Fund award (to S. R. P.) and by National Institutes of Health Grant HL55487 (to A. D.) The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a Heart and Stroke Foundation of Canada fellowship, and current holder of an American Heart Association (Ohio Valley Affiliate) fellowship.

‖ To whom correspondence should be addressed: Dept. of Pharmacology, University of Kentucky Medical Center, MS305, Lexington, KY 40536-0298. Tel.: 859-323-3996 (ext. 293); Fax: 859-257-9166; E-mail: spost@pop.uky.edu.

[1] The abbreviations used are: M-CSF, macrophage colony-stimulating factor; β-VLDL, β-very low density lipoprotein; LDL, low density lipoprotein; AcLDL, acetylated low density lipoproteins; SR-A, class A scavenger receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BSA, bovine serum albumin; PI 3-kinase, phosphatidylinositol 3-kinase.

have shown that M-CSF deficiency greatly reduced lesion development in the atherogenic susceptible LDL receptor (−/−) (19) and apolipoprotein E (−/−) strains (20–22). Interestingly, findings in the study by Rajavashisth *et al.* (19) suggest the effect of M-CSF deficiency on atherogenesis was not a consequence of a reduction in circulating monocytes, but resulted from a direct effect of cytokine deficiency on lesion formation. In contrast to studies using op/op mice, intravenous injection of Watanabe heritable hyperlipidemic rabbits with M-CSF (three times a week for 8.5 months) significantly reduced cholesterol ester content and size of aortic atherosclerotic lesions (23). Thus, the role of M-CSF in atherosclerosis may be complex and dependent upon the extent and the site of M-CSF expression.

A number of *in vitro* studies have shown that prolonged exposure (>24 h) of macrophages to M-CSF enhanced the uptake of modified-LDL via both nonspecific (24) and specific pathways; the latter involving enhanced SR-A expression (25–27). Because binding of M-CSF to its receptor activates several signaling pathways (11), we examined whether a short exposure of macrophages to M-CSF would alter the rapid metabolism of lipoprotein-cholesterol. In contrast to prolonged M-CSF exposure, our results indicate that a brief exposure (5 h) of macrophages to recombinant M-CSF markedly increases the uptake of certain lipoprotein particles. Specifically, we found that M-CSF treatment enhanced the uptake of both LDL and β-VLDL, obtained from cholesterol-fed rabbits, but did not affect the uptake of AcLDL. In addition, we have shown that enhanced β-VLDL metabolism following M-CSF treatment involves activation of a $G_{i/o}$ protein signaling pathway that regulates LDL receptor-mediated lipoprotein metabolism.

EXPERIMENTAL PROCEDURES

Chemicals—Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and high glucose, DMEM with 25 mM HEPES but without sodium bicarbonate, and heat-inactivated fetal bovine serum (FBS) were purchased from Life Technologies, Inc. Penicillin and streptomycin were purchased from Sigma. Recombinant murine M-CSF was purchased from R&D Systems (Minneapolis, MN) and was solubilized in phosphate-buffered saline containing 0.1% bovine serum albumin (BSA; Sigma). Pertussis toxin (*Bordetella pertussis*) and mastoparan were from Calbiochem (La Jolla, CA) and were both solubilized in phosphate-buffered saline containing 0.1% BSA. Wortmannin was purchased from Sigma and solubilized as a stock solution in dimethyl sulfoxide (Me_2SO). $Na^{125}I$ and [^{3}H]oleate were obtained from Amersham Pharmacia Biotech.

Lipoprotein Isolation, Acetylation, and Radioiodination—LDL (d = 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation (28) of EDTA-anticoagulated plasma obtained from healthy normolipidemic volunteers. LDL was dialyzed against saline containing 1 mM EDTA (pH 7.4). AcLDL was prepared by chemical modification of the LDL with acetic anhydride as described by Basu *et al.* (29) and confirmed by comparing the relative electrophoretic mobility of AcLDL to native LDL on an agarose gel. β-VLDL (d = 1.006–1.019 g/ml) was isolated by sequential ultracentrifugation of EDTA-anticoagulated plasma obtained from 12-h fasted male New Zealand White rabbits (Myrtles) maintained on a cholesterol-enriched diet (1% w/w cholesterol; test diet 9456, Purina, Richmond, IN). 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 (30). β-VLDL and LDL were radiolabeled using an indirect labeling method with $Na^{125}I$ using IODO-GEN® pre-coated tubes (Pierce) following manufacturer's instructions; in this method, the scavenging wash step was omitted and the radioiodination reaction was terminated by passing the sample over two desalting columns (Bio-Gel P6DG, Pierce).

Cell Culture—J774A.1 cells, a murine macrophage cell line, was obtained from American Type Culture Collection (Manassas, VA) and were maintained in DMEM containing penicillin (10 units/ml), streptomycin (10 μg/ml), and 10% FBS. Resident macrophages were collected from both male and female inbred C57BL/6 wild type and LDL receptor-deficient mice (Jackson Laboratory, Bar Harbor, ME) and male outbred Cr:NIH (S) Swiss mice (National Cancer Institute, Charles River Laboratories, Frederick, MD) by peritoneal lavage with ice-cold sterile saline. Cells were resuspended in DMEM containing antibiotics and FBS. After an overnight incubation at 37 °C, non-adherent peritoneal cells were removed by gently washing cells three times with serum-free DMEM. Adherent peritoneal macrophages were then cultured in medium with FBS.

Cholesterol Esterification Assay—The incorporation of [^{3}H]oleic acid into cholesterol esters was used as a measure of macrophage-mediated uptake of lipoproteins. Lipoproteins were added to either J774A.1 or peritoneal macrophages and incubated for 5 h in DMEM plus antibiotics but no serum. Prior to the addition of lipoproteins, macrophages were treated with pertussis toxin (100 ng/ml) for 24 h, with wortmannin (100 nM) for 45 min and with M-CSF (1–100 ng/ml) or mastoparan (10 μM) for 15 min. In addition, each well of cells received 0.9 μCi [^{3}H]oleic acid complexed with fatty acid-free BSA in a molar ratio of 5:1. The cholesterol ester assays were analyzed as described previously (15).

^{125}I-β-VLDL Specific Association and Degradation by Macrophages—To quantify lipoprotein association and degradation, J774A.1 macrophages were cultured in 12-well plates in the presence of DMEM plus antibiotics and serum. When the cells were 80% confluent, the medium was changed to 0.5 ml/well of serum-free DMEM plus antibiotics and 0.5% BSA, and the macrophages were cultured for an additional 5 h in the absence or presence of M-CSF (25 ng/ml) and 50 μg ^{125}I-β-VLDL. Culture plates were put on ice and the incubation medium was transferred to disposable glass 12 × 75-mm tubes containing 55 μl of 100% trichloroacetic acid. The cells were then washed once with ice-cold buffer A (154 mM NaCl, 42 mM Tris-HCl, 8 mM Tris, 0.2% BSA, pH 7.4) and twice with BSA-free buffer A. Cellular protein was solubilized for 16 h at room temperature in 1 ml of 0.1 N NaOH. Tubes containing the medium-trichloroacetic acid solution were vortexed, placed on ice for a minimum of 30 min, and subjected to centrifugation (1500 × g, 30 min, 4 °C). Radioactivity in the supernatant of the media following trichloroacetic acid precipitation and in the protein extract were determined using a CliniGamma 1272 [gamma- counter (Wallac Oy, Turku, Finland); cell protein was determined using the Bio-Rad protein assay with BSA used to generate a standard curve. The amount of associated ^{125}I-β-VLDL and degradation products generated in the absence of cells was also measured and subtracted from the corresponding samples incubated with cells. Degraded and cell-associated ^{125}I-β-VLDL was expressed as nanograms of ^{125}I-β-VLDL/mg of cell protein/5 h.

^{125}I-β-VLDL and ^{125}I-LDL Specific Binding to Macrophages—To quantify lipoprotein binding, J774A.1 macrophages were cultured in 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 5 h in the absence or presence of M-CSF (25 ng/ml). Prior to beginning the binding study, the cell media was replaced with ice-cold DMEM supplemented with 0.5% BSA and 25 mM HEPES; pH 7.4. Following an additional 0.5 h at 4 °C, either ^{125}I-β-VLDL (0.25–20 μg/ml) or ^{125}I-LDL (0.25–80 μg/ml) was added to the cells in the absence or presence of a 20-fold excess of unlabeled β-VLDL or LDL, respectively, and the cells were then incubated for an additional 2.5 h at 4 °C. The cells were then washed once with ice-cold buffer A plus 0.2% BSA and twice with BSA-free buffer A. Cellular protein was solubilized for 16 h at room temperature in 0.5 ml of 0.1 N NaOH. Radioactivity in the protein extract was determined as stated above; cell protein was determined using the Bio-Rad protein assay. The amount of specific binding was calculated by subtracting the amount of ^{125}I-lipoprotein bound in the presence of a 20-fold excess of unlabeled lipoprotein from the total amount of ^{125}I-lipoprotein bound. The results are expressed as nanograms of ^{125}I-β-VLDL or ^{125}I-LDL bound/mg of cell protein.

Statistical Analysis—Data analysis was performed using SigmaStat 2.03 software (SPSS Inc.). For each parameter, the mean and standard error of mean (S.E.) were calculated. Differences between a control and single experimental group were evaluated by t test. In those experiments where more than one experimental group existed (Figs. 1, 7, and 8*A*), differences were evaluated using a one-way analysis of variance with all pairwise multiple comparison procedures conducted using the Tukey test. Values with $p < 0.05$ were considered statistically significant.

RESULTS

M-CSF Treatment Enhances the Metabolism of β-VLDL and LDL—Prolonged exposure (>24 h) of monocyte-derived macrophages to M-CSF has been shown to regulate the metabolism of modified-LDL, but not β-VLDL (25, 27, 31). In the current study, we examined whether acute treatment of macrophages with M-CSF would differ from that of prolonged M-CSF expo-

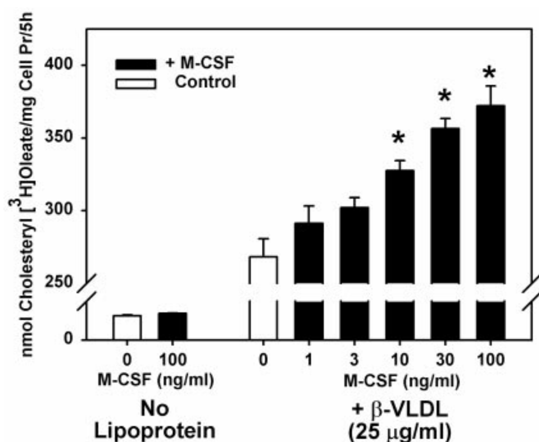

FIG. 1. **Cholesterol esterification was determined in mouse peritoneal macrophages that were untreated (*open bars*) or treated (*solid bars*) for 15 min with increasing concentrations of recombinant mouse M-CSF (1–100 ng/ml) prior to incubation with 25 μg of protein/ml of β-VLDL and a [3H]oleic acid-albumin complex for 5 h.** Cellular esterified cholesterol was isolated by thin layer chromatography and cholesteryl [3H]oleate quantified by liquid scintillation counting. Values represent mean ± S.E. of three separate determinations made in triplicate. *, $p = 0.001$ *versus* β-VLDL alone.

sure by having a significant affect on the metabolism of β-VLDL. Using incorporation of [3H]oleic acid into cholesterol esters as a measure of macrophage-mediated lipoprotein metabolism, we found that acute exposure of peritoneal macrophages to M-CSF (10–100 ng/ml) significantly enhanced β-VLDL-induced cholesterol ester deposition (268.2 ± 12.3 *versus* 372.2 ± 13.7 nmol/mg of protein/5 h at 100 ng/ml of M-CSF; $p < 0.001$, $n = 3$, Fig. 1). Maximal enhancement of β-VLDL metabolism by M-CSF treatment occurred at a concentration of approximately 25 ng/ml (Fig. 1). Similarly, M-CSF, in a concentration dependent manner, enhanced the metabolism of β-VLDL by the murine macrophage cell line J774A.1 (data not shown). Metabolism of β-VLDL by peritoneal and J774A.1 macrophages, in either the absence or presence of M-CSF, saturated at a concentration of 25 μg of lipoprotein/ml of medium, indicating that accumulation of this lipoprotein (± M-CSF) involved a specific receptor-mediated uptake process (Fig. 2, *A* and *B*). Similar to β-VLDL, native LDL-induced cholesterol ester deposition was significantly enhanced by acute M-CSF treatment of J774A.1 macrophages (Fig. 2*C*).

M-CSF Treatment Enhances the Association and Degradation of β-VLDL—To examine whether cytokine treatment directly enhancing internalization and degradation of β-VLDL, association and degradation studies were conducted at 37 °C using 125I-β-VLDL and J774A.1 macrophages incubated in the absence or presence of M-CSF (25 ng/ml). Compared with nontreated cells, M-CSF treatment significantly enhanced the specific degradation of β-VLDL by macrophages at 37 °C (2392.3 ± 473.2 *versus* 3785.9 ± 358.2 ng/mg of protein/5 h; at 50 μg of protein/ml, $p = 0.018$, $n = 5$) (Fig. 3*A*). In parallel, cell association of 125I-β-VLDL was also enhanced by M-CSF treatment (212.5 ± 14.1 *versus* 296.1 ± 37.7 ng/mg of protein/5 h; at 50 μg of protein/ml, $p = 0.047$, $n = 5$) (Fig. 3*B*).

M-CSF Treatment Does Not Enhance AcLDL Metabolism—To determine whether the effect of M-CSF was specific for β-VLDL, mouse peritoneal macrophages were incubated with AcLDL to examine scavenger receptor-mediated lipoprotein metabolism. In the absence of M-CSF, incubation of macrophages with AcLDL caused significant cholesterol ester deposition compared with control cells (245 ± 16 *versus* 12 ± 3 nmol/mg of protein/5 h; $p = 0.0001$, $n = 3$, Fig. 4). In contrast to β-VLDL, addition of M-CSF (25 ng/ml) did not alter cholesterol ester deposition during incubation with AcLDL (249 ± 6 nmol/mg of protein/5 h; p = non-significant *versus* incubation with AcLDL alone, $n = 3$, Fig. 4). This result suggests that the enhanced uptake of β-VLDL observed in the presence of M-CSF did not result from a generalized effect on lipoprotein uptake and metabolism.

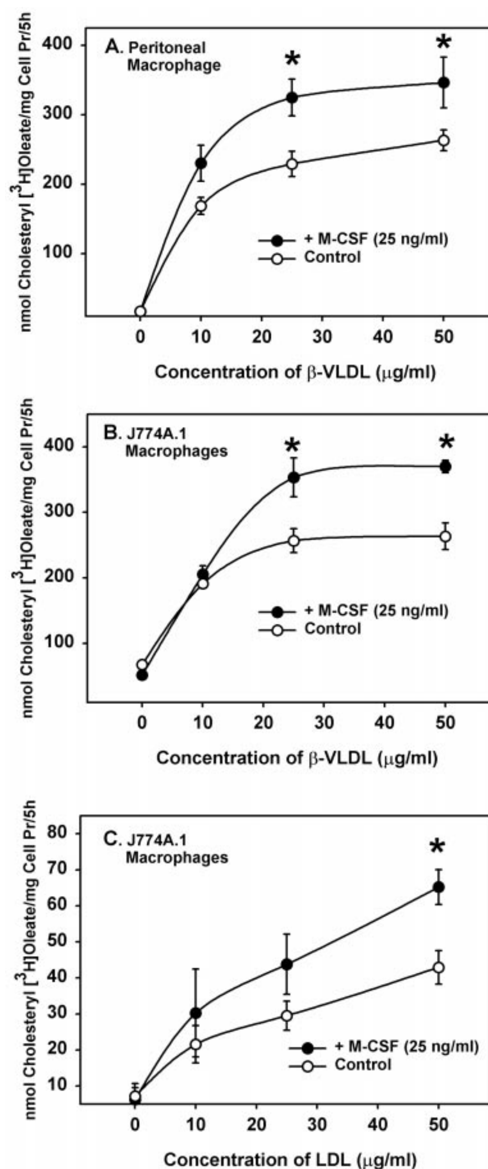

FIG. 2. **Cholesterol esterification was determined in mouse peritoneal macrophages (*A*) or cultured J774A.1 murine macrophages (*B* and *C*), that were untreated (*open circles*) or treated (*solid circles*) for 15 min with recombinant mouse M-CSF (25 ng/ml) prior to incubation with increasing concentrations of either β-VLDL or LDL (10–50 μg of protein/ml) and a [3H]oleic acid-albumin complex for 5 h.** Cellular esterified cholesterol was isolated by thin layer chromatography and cholesteryl [3H]oleate quantified by liquid scintillation counting. Values represent mean ± S.E. of three determinations made in triplicate. *A*, *, $p = 0.041$; *B*, *, $p = 0.05$; *C*, *, $p = 0.0057$.

Enhanced β-VLDL Metabolism Is Mediated by the LDL Receptor—β-VLDL has the potential to be internalized by all members of the LDL receptor family (32). However, β-VLDL, either in the presence or absence of M-CSF, failed to simulate cholesterol ester deposition in peritoneal macrophages isolated from LDL receptor (−/−) mice (Fig. 5). These results indicate that M-CSF enhances β-VLDL metabolism via a process that specifically involves the LDL receptor.

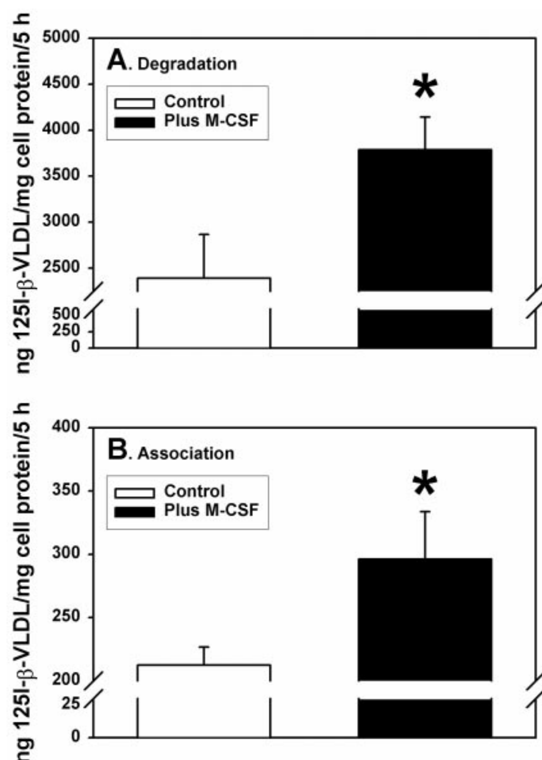

FIG. 3. **J774A.1 macrophages were incubated in the absence (*open bars*) or presence (*solid bars*) of M-CSF (25 ng/ml) and [^{125}I]β-VLDL (50 μg of protein/ml) for 5 h at 37 °C.** *A*, specific degradation of ^{125}I-β-VLDL; *B*, cell association of ^{125}I-β-VLDL. Values represent mean ± S.E. of five determinations made in triplicate. *, $p \leq 0.05$ *versus* incubation in the absence of M-CSF.

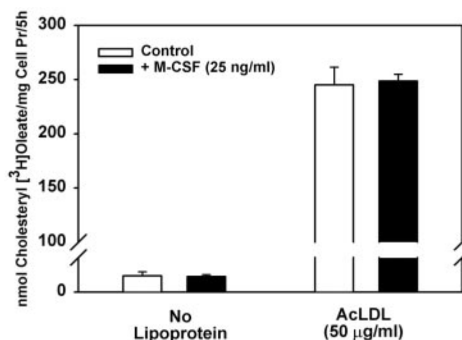

FIG. 4. **Cholesterol esterification was determined in mouse peritoneal macrophages that were untreated (*open bars*) or treated (*solid bars*) for 15 min with recombinant mouse M-CSF (25 ng/ml) prior to incubation with 50 μg of protein/ml of AcLDL and a [^{3}H]oleic acid-albumin complex for 5 h.** Cellular esterified cholesterol was isolated by thin layer chromatography and cholesteryl [^{3}H]oleate quantified by liquid scintillation counting. Values represent mean ± S.E. of three separate determinations made in triplicate.

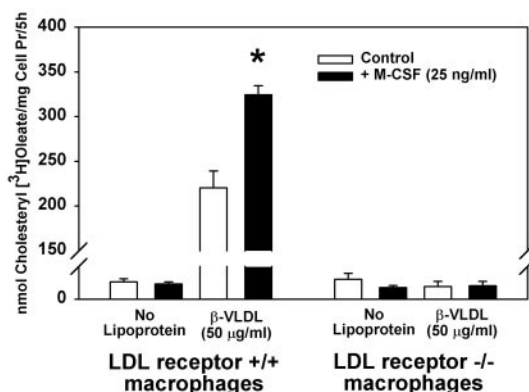

FIG. 5. **Cholesterol esterification was determined in mouse peritoneal macrophages derived from either wild type (LDLr+/+) or LDL receptor-deficient (LDLr−/−) mice.** Macrophages were untreated (*open bars*) or treated (*solid bars*) for 15 min with recombinant mouse M-CSF (25 ng/ml) prior to incubation with 50 μg of protein/ml of β-VLDL and a [^{3}H]oleic acid-albumin complex for 5 h. Cellular esterified cholesterol was isolated by thin layer chromatography and cholesteryl [^{3}H]oleate quantified by liquid scintillation counting. Values represent mean ± S.E. of three separate determinations made in triplicate. *, $p = 0.008$.

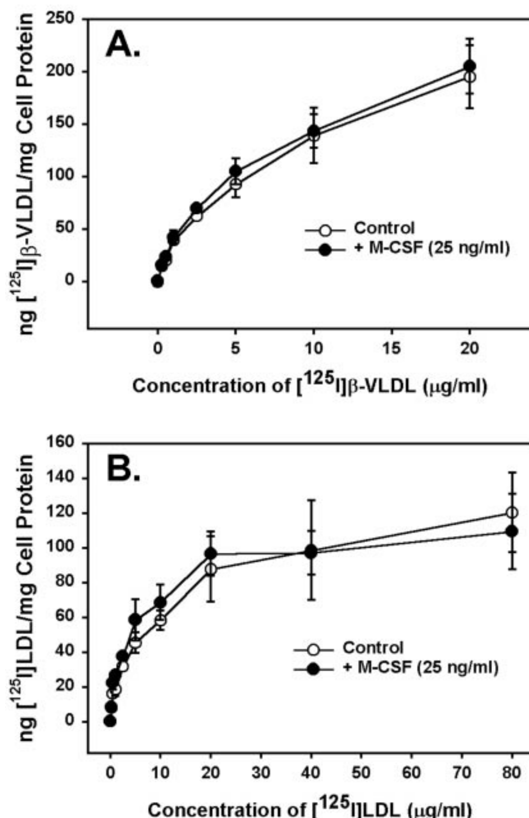

FIG. 6. **J774A.1 macrophages were incubated in the absence (*open circles*) or presence (*solid circles*) of M-CSF (25 ng/ml) for 5 h at 37 °C.** The cells were rinsed and incubated with increasing concentration of ^{125}I-β-VLDL (0.25–20 μg of protein/ml) (*A*) or ^{125}I-LDL (0.25–80 μg of protein/ml) (*B*) at 4 °C for 2.5 h. The amount of specific binding was calculated by subtracting the amount of ^{125}I-lipoprotein bound in the presence of a 20-fold excess of unlabeled lipoprotein from the total amount of ^{125}I-lipoprotein bound. Values represent mean ± S.E. of either three (^{125}I-β-VLDL) or two (^{125}I-LDL) determinations made in duplicate.

M-CSF Treatment Does Not Increase β-VLDL or LDL Binding—To examine whether M-CSF treatment enhanced the number of β-VLDL binding sites at the cell surface, binding studies were conducted at 4 °C (to prevent ligand internalization) using ^{125}I-β-VLDL and J774A.1 macrophages incubated in the absence or presence of M-CSF (25 ng/ml). M-CSF treatment did not enhance the specific binding of β-VLDL by macrophages at 4 °C (Fig. 6*A*). Similarly, M-CSF treatment did not increase the binding of native ^{125}I-LDL to its receptor at 4 °C (Fig. 6*B*). In contrast to both ^{125}I-β-VLDL and ^{125}I-LDL binding at 4 °C, M-CSF treatment of J774A.1 macrophages at 37 °C significantly enhanced the metabolism of both β-VLDL (263.4 ± 20.4 *versus* 369.9 ± 9.3 nmol/mg of protein/5 h; at 50 μg of protein/ml, $p = 0.009$, $n = 3$) and LDL (42.9 ± 4.6 *versus* 65.2 ± 4.8 nmol/mg of protein/5 h; at 50 μg of protein/ml, $p = 0.0057$, $n = 2$) (Fig. 2, *B* and *C*).

M-CSF Does Not Enhance β-VLDL Metabolism via PI 3-Ki-

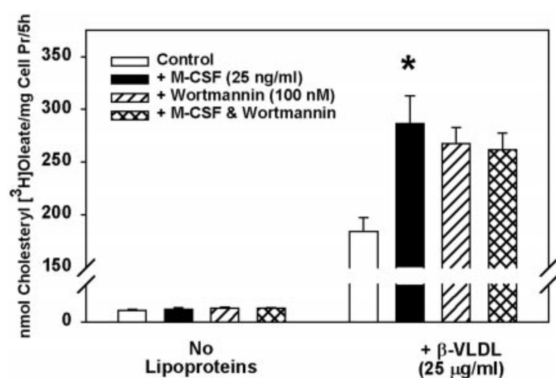

FIG. 7. **Cholesterol esterification was determined in mouse peritoneal macrophages that were untreated (*open* and *solid bars*) or treated (*diagonal* and *hatched bars*) with a specific PI 3-kinase inhibitor, wortmannin (100 nM), for 30 min and then incubated for 15 min in the absence (*open* and *diagonal bars*) or presence (*solid* and *hatched bars*) of recombinant mouse M-CSF (25 ng/ml).** β-VLDL (25 μg of protein/ml) and a [^{3}H]oleic acid-albumin complex was added and incubations continued for 5 h. Cellular esterified cholesterol was isolated by thin layer chromatography and cholesteryl [^{3}H]oleate quantified by liquid scintillation counting. Values represent mean ± S.E. of three separate determinations made in triplicate. *, $p = 0.02$ *versus* incubation with β-VLDL alone.

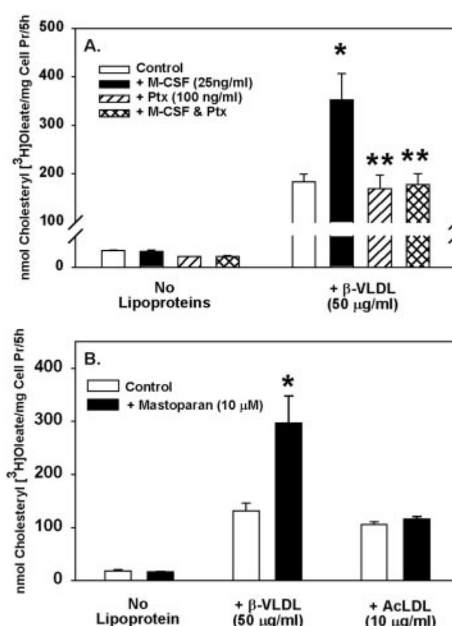

FIG. 8. *A*, cholesterol esterification was determined in mouse peritoneal macrophages that were untreated (*open* and *solid bars*) or treated (*diagonal* and *hatched bars*) with pertussis toxin (*Ptx*, 100 ng/ml) for 24 h and then incubated for 15 min in the absence (*open* and *diagonal bars*) or presence (*solid* and *hatched bars*) of recombinant mouse M-CSF (25 ng/ml), prior to incubation with β-VLDL (50 μg of protein/ml) and a [^{3}H]oleic acid-albumin complex for 5 h. *B*, cholesterol esterification was determined in mouse peritoneal macrophages that were untreated (*open bars*) or treated (*solid bars*) for 15 min with mastoparan (10 μM) prior to incubation with β-VLDL (50 μg of protein/ml), AcLDL (10 μg of protein/ml), and a [^{3}H]oleic acid-albumin complex for 5 h. Cellular esterified cholesterol was isolated by thin layer chromatography and cholesteryl [^{3}H]oleate quantified by liquid scintillation counting. Values represent mean ± S.E. of three separate determinations (all groups in *A* and AcLDL group in *B*) or four separate determinations (β-VLDL group in *B*) made in triplicate. *A*, *, $p = 0.014$ *versus* incubation with β-VLDL alone, ** $p = 0.014$ *versus* incubation with β-VLDL + M-CSF. *B*, *, $p = 0.015$ *versus* incubation with β-VLDL alone.

nase or Src Kinase—Binding of M-CSF to its receptor initiates a number of specific signaling cascades, including activation of PI 3-kinase, the non-receptor Src family of tyrosine kinases, and $G_{i/o}$ proteins (11). To examine the potential role of PI 3-kinase activation in M-CSF-enhanced β-VLDL uptake, a specific PI 3-kinase inhibitor, wortmannin, was added prior to the addition of β-VLDL alone or β-VLDL plus M-CSF. Wortmannin irreversibly inhibits the catalytic subunit of mammalian PI 3-kinase with an $IC_{50} = 3$ nM (33–35). Specific inhibition of this kinase signaling pathway with wortmannin up to 100 nM did not affect the enhanced metabolism of β-VLDL following M-CSF-treatment (Fig. 7). Similarly, the Src tyrosine kinase inhibitors 4-amino-5-(-4-cholorophenyl)-7-(*t*-butyl)pyrazolo 3,4-*d*-pyrimidine (PP2; 100 nM) and herbimycin A (1 μM) did not affect the enhanced metabolism of β-VLDL mediated by M-CSF treatment (data not shown).

M-CSF Enhances β-VLDL Metabolism via $G_{i/o}$ Protein Activation—To examine the role of heterotrimeric G proteins, peritoneal macrophages were treated with either a specific inhibitor of $G_{i/o}$ protein signaling, pertussis toxin (36, 37), or a direct $G_{i/o}$ protein activator, mastoparan (38), prior to the addition of β-VLDL alone or β-VLDL plus M-CSF. As shown previously (15), pertussis toxin treatment did not affect cholesterol ester deposition during incubation with β-VLDL alone (183.0 ± 16.7 *versus* 169.2 ± 28.7 nmol/mg of protein/5 h; p = non-significant, $n = 3$) (Fig. 8*A*). However, pre-treatment with toxin completely attenuated the augmented cholesterol ester deposition promoted by M-CSF (352.0 ± 55.0 *versus* 177.4 ± 22.5 nmol/mg of protein/5 h; β-VLDL+M-CSF *versus* β-VLDL + M-CSF + toxin, $p = 0.014$, $n = 3$) (Fig. 8*A*). Treatment of peritoneal macrophages with mastoparan was found to mimic the effect of M-CSF; metabolism of β-VLDL was significantly enhanced in the presence of mastoparan (130.9 ± 14.8 *versus* 295.5 ± 51.4 nmol/mg of protein/5 h; $p = 0.015$, $n = 5$), whereas AcLDL metabolism was not affected (105.3 ± 5.5 *versus* 115.1 ± 5.4 nmol/mg of protein/5 h; p = non-significant, $n = 3$) (Fig. 8*B*).

DISCUSSION

We examined whether β-VLDL metabolism by macrophages would be affected by a brief exposure of the cells to M-CSF. Acute M-CSF treatment of both primary isolates and cultured murine macrophages significantly enhanced cholesterol ester deposition upon incubation of cells with either LDL or β-VLDL. In contrast to its effect on metabolism of LDL receptor ligands, M-CSF did not alter cholesterol ester deposition in macrophages treated with AcLDL. Thus, the effect of M-CSF-treatment on β-VLDL- and LDL-mediated cholesterol ester deposition was not the result of a nonspecific affect on lipoprotein uptake and metabolism. To further address this issue, we performed uptake and degradation experiments at 37 °C using ^{125}I-β-VLDL. M-CSF treatment significantly enhanced degradation of ^{125}I-β-VLDL consistent with the notion that the cytokine was specifically enhancing lipoprotein uptake rather than affecting cholesterol metabolism downstream of lysosomal degradation of the lipoprotein. Enhanced metabolism of both β-VLDL and LDL induced by M-CSF treatment was found to be saturable, indicating that accumulation of these lipoproteins involved a specific uptake process. However, 4 °C binding studies using both ^{125}I-LDL and ^{125}I-β-VLDL indicated that M-CSF did not enhance lipoprotein metabolism via increasing cell surface lipoprotein receptor (LDL receptor) expression. In contrast to our findings, prolonged exposure (4 days) of a human monocyte-derived macrophage cell line (tetradecanoyl phorbol acetate-treated THP-1cells) to M-CSF had no affect on β-VLDL-induced cholesterol ester deposition (31). Possible explanations to account for this discrepancy may include desensitization of an M-CSF signaling pathway in response to prolonged incubation with the cytokine, or changes in an M-CSF signaling pathway following prolonged exposure to the phorbol ester used to differentiate THP-1 monocytes.

A number of studies have shown that prolonged exposure of macrophages to M-CSF will lead to an enhanced uptake of modified LDL via both nonspecific (24) and specific pathways; the latter involving enhanced SR-A expression (25–27). In contrast, we found that acute treatment of peritoneal macrophages with M-CSF did not affect the uptake of AcLDL, which binds to SR-A but not the LDL receptor, suggesting that the effect of M-CSF did not reflect generalized enhancement of lipoprotein uptake and metabolism. A likely explanation to account for the contrast between our findings and those of previous studies is that 5 h of M-CSF exposure is not sufficient to significantly alter SR-A receptor expression and function.

Incubation of peritoneal macrophages derived from LDL receptor (−/−) mice with β-VLDL did not increase cholesterol ester deposition over basal concentrations. Furthermore, treatment of LDL receptor (−/−) peritoneal macrophages with M-CSF did not augment β-VLDL uptake. These results strongly implicate the LDL receptor in mediating β-VLDL metabolism by peritoneal macrophages and that M-CSF enhances β-VLDL metabolism by affecting LDL receptor activity. Despite the fact that uptake of β-VLDL was found to occur via the LDL receptor, M-CSF treatment of LDL receptor (+/+) macrophages did not increase binding of either ^{125}I-β-VLDL or ^{125}I-LDL to the cell surface. Consistent with our finding, Stopeck *et al.* (39) previously demonstrated that 24-h pretreatment of the human hepatic cell line, HepG2, with M-CSF did not affect binding of ^{125}I-LDL to the LDL receptor at 4 °C. Together, these data indicate that the increase in β-VLDL metabolism observed in M-CSF-treated cells does not result from increased expression or changes in the number of cell surface receptors for β-VLDL or LDL.

Pinocytosis is an active endocytic process that is important in the uptake of extracellular fluid (fluid phase pinocytosis) and macromolecules (receptor-mediated endocytosis), and in the turnover of the plasma membrane and its components (40, 41). Unlike receptor-mediated endocytosis, which exclusively involves internalization via clathrin-coated vesicles, fluid phase pinocytosis, which includes both macro- and micro-pinocytosis, can occur through either clathrin-coated or uncoated vesicles (42). M-CSF treatment has been shown to rapidly stimulate fluid-phase pinocytosis in murine bone marrow derived macrophages via activation of PI 3-kinase (43, 44). In contrast to its effect on fluid phase pinocytosis, M-CSF treatment did not enhance receptor-mediated endocytosis of either transferrin or AcLDL via the transferrin receptor or SR-A, respectively (44). The PI 3-kinase inhibitors wortmannin and LY294002 prevented enhancement of fluid phase pinocytosis, but had little effect on receptor-mediated endocytosis of transferrin (44) and AcLDL (44, 45). Our findings that pre-treatment of peritoneal macrophages with wortmannin did not inhibit the enhanced uptake of β-VLDL mediated by M-CSF is in agreement with earlier studies, which showed that M-CSF does not affect receptor-mediated endocytosis, and argues strongly in favor of the idea that M-CSF enhances uptake of β-VLDL via a receptor-mediated endocytic process and not via activation of a PI 3-kinase-mediated macropinocytic process.

Pertussis toxin, a specific inhibitor of $G_{i/o}$ protein signaling (36, 37), inhibits signal transduction and proliferation of monocytes and macrophages stimulated with M-CSF (46–50), suggesting that $G_{i/o}$ proteins are involved in M-CSF-mediated signal transduction. Subsequent to these earlier findings, Corre and Hermouet (51) showed that $G\alpha_{i2}$ is the G protein subtype responsible for the effects of pertussis toxin on M-CSF stimulated cellular proliferation of BAC 1.2F5 cells, a murine macrophage cell line that is dependent on M-CSF for its survival and proliferation. Our results present strong evidence showing that enhanced uptake of β-VLDL upon M-CSF treatment is mediated by $G_{i/o}$ protein signaling. First, pre-treatment with pertussis toxin did not affect cholesterol ester deposition during incubation with β-VLDL alone; however, pre-treatment with toxin completely attenuated the augmented cholesterol ester deposition promoted by M-CSF. Second, acute treatment with mastoparan, a direct activator of $G_{i/o}$ proteins (38) mimicked M-CSF by enhancing the metabolism of β-VLDL, but not that of AcLDL.

In summary, acute incubation of peritoneal macrophages with M-CSF has pronounced effects on the metabolism of β-VLDL and native LDL. The findings from this study describe a novel regulatory pathway linking $G_{i/o}$ protein-mediated signaling and LDL receptor-mediated lipoprotein metabolism. Furthermore, by showing that signaling via the M-CSF receptor promotes rapid cholesterol ester accumulation, this study defines a potentially important control mechanism for regulating lipoprotein metabolism and atherogenesis.

Acknowledgment—We thank Debra Rateri for reviewing the manuscript.

REFERENCES

1. Rambaldi, A., Young, D. C., and Griffin, J. D. (1987) *Blood* **69,** 1409–1413
2. Rajavashisth, T. B., Andalibi, A., Territo, M. C., Berliner, J. A., Navab, M., Fogelman, A. M., and Lusis, A. J. (1990) *Nature* **344,** 254–257
3. Sieff, C. A., Niemeyer, C. M., Mentzer, S. J., and Faller, D. V. (1988) *Blood* **72,** 1316–1323
4. Hallet, M., Praloran, V., Vie, H., Peyrat, M., Wong, G., Witek-Giannotti, J., Soulillou, J., and Moreau, J. (1991) *Blood* **77,** 780–786
5. Tushinski, R. J., Oliver, I. T., Guilbert, L. J., Tynan, P. W., Warner, J. R., and Stanley, E. R. (1982) *Cell* **28,** 71–81
6. Stanley, E. R., Guilbert, L. J., Tushinski, R. J., and Bartelmez, S. H. (1983) *J. Cell. Biochem.* **21,** 151–159
7. Sherr, C. J. (1988) *Biochim. Biophys. Acta* **948,** 225–243
8. Sherr, C. J., Rettenmier, C. W., and Roussel, M. F. (1988) *Cold Spring Harbor Symp. Quant. Biol.* **53,** 521–530
9. Sherr, C. J., Rettenmier, C. W., Sacca, R., Roussel, M. F., Look, A. T., and Stanley, E. R. (1985) *Cell* **41,** 665–676
10. Sacca, R., Stanley, E. R., Sherr, C. J., and Rettenmier, C. W. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 3331–3335
11. Hamilton, J. A. (1997) *J. Leukocyte Biol.* **62,** 145–155
12. Sano, H., Higashi, T., Matsumoto, K., Melkko, J., Jinnouchi, Y., Ikeda, K., Ebina, Y., Makino, H., Smedsrod, B., and Horiuchi, S. (1998) *J. Biol. Chem.* **273,** 8630–8637
13. Meng, F., and Lowell, C. A. (1998) *EMBO J.* **17,** 4391–4403
14. Goretzki, L., and Mueller, B. M. (1998) *Biochem. J.* **336,** 381–386
15. Whitman, S. C., Daugherty, A., and Post, S. R. (2000) *J. Lipid Res.* **41,** 807–813
16. Clinton, S. K., Underwood, R., Hayes, L., Sherman, M. L., Kufe, D. W., and Libby, P. (1992) *Am. J. Pathol.* **140,** 301–316
17. Rosenfeld, M. E., Yla-Herttuala, S., Lipton, B. A., Ord, V. A., Witztum, J. L., and Steinberg, D. (1992) *Am. J. Pathol.* **140,** 291–300
18. Inaba, T., Yamada, N., Gotoda, T., Shimano, H., Shimada, M., Momomura, K., Kadowaki, T., Motoyoshi, K., Tsukada, T., Morisaki, N., Saito, Y., Yoshida, S., Takaku, F., and Yazaki, Y. (1992) *J. Biol. Chem.* **267,** 5693–5699
19. Rajavashisth, T., Qiao, J., Tripathi, S., Tripathi, J., Mishra, N., Hua, M., Wang, X., Loussararian, A., Clinton, S., Libby, P., and Lusis, A. (1998) *J. Clin. Invest.* **101,** 2702–2710
20. Smith, J. D., Trogan, E., Ginsberg, M., Grigaux, C., Tian, J., and Miyata, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8264–8268
21. Qiao, J., Tripathi, J., Mishra, N. K., Cai, Y., Tripathi, S., Wang, X., Imes, S., Fishbein, M. C., Clinton, S. K., Libby, P., Lusis, A. J., and Rajavashisth, T. B. (1997) *Am. J. Pathol.* **150,** 1687–1699
22. de Villiers, W. J. S., Smith, J. D., Miyata, M., Dansky, H. M., Darley, E., and Gordon, S. (1998) *Arterioscler. Thromb. Vasc. Biol.* **18,** 631–640
23. Inoue, I., Inaba, T., Motoyoshi, K., Harada, K., Shimano, H., Kawamura, M., Gotoda, T., Oka, T., Shiomi, M., Watanabe, Y., Tsukada, T., Yazaki, Y., Takaku, F., and Yamada, N. (1992) *Atherosclerosis* **93,** 245–254
24. Jessup, W., Squires, B., Kritharides, L., Hume, D. A., and Dean, R. T. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17,** 18–25
25. Ishibashi, S., Inaba, T., Shimano, H., Harada, K., Inoue, I., Mokuno, H., Mori, N., Gotoda, T., Takaku, F., and Yamada, N. (1990) *J. Biol. Chem.* **265,** 14109–14117
26. de Villiers, W. J. S., Fraser, I. P., Hughes, D. A., Doyle, A. G., and Gordon, S. (1994) *J. Exp. Med.* **180,** 705–709
27. de Villiers, W. J. S., Fraser, I. P., and Gordon, S. (1994) *Immunol. Lett.* **43,** 73–79
28. Havel, R., Eder, H. A., and Bragdon, J. H. (1955) *J. Clin. Invest.* **34,** 1345–1353
29. Basu, S. K., Goldstein, J. L., Anderson, R. G. W., and Brown, M. J. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 3178–3182
30. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
31. Ishii, I., Yanagimachi, M., Shirai, K., Saito, Y., and Hirose, S. (1994) *Biochim. Biophys. Acta* **1212,** 278–284

32. Hussain, M. M., Strickland, D. K., and Bakillah, A. (1999) *Annu. Rev. Nutr.* **19,** 141–172
33. Arcaro, A., and Wymann, M. P. (1993) *Biochem. J.* **296,** 297–301
34. Thelen, M., Wymann, M. P., and Langen, H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 4960–4964
35. Yano, H., Agatsuma, T., Nakanishi, S., Saitoh, Y., Fukui, Y., Nonomura, Y., and Matsuda, Y. (1995) *Biochem. J.* **312,** 145–150
36. Katada, T., and Ui, M. (1982) *J. Biol. Chem.* **257,** 7210–7216
37. Katada, T., and Ui, M. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 3129–3133
38. Higashijima, T., Uzu, S., Nakajima, T., and Ross, E. M. (1988) *J. Biol. Chem.* **263,** 6491–6494
39. Stopeck, A. T., Nicholson, A. C., Mancini, F. P., and Hajjar, D. P. (1993) *J. Biol. Chem.* **268,** 17489–17494
40. Silverstein, S. C., Steinman, R. M., and Cohn, Z. A. (1977) *Annu. Rev. Biochem.* **46,** 669–722
41. Mellman, I. (1996) *Annu. Rev. Cell Dev. Biol.* **12,** 575–625
42. Swanson, J. A., and Baer, S. C. (1995) *Trends Cell Biol.* **5,** 89–93
43. Racoosin, E. L., and Swanson, J. A. (1989) *J. Exp. Med.* **170,** 1635–1648
44. Racoosin, E. L., and Swanson, J. A. (1992) *J. Cell Sci.* **102,** 867–880
45. Araki, N., Johnson, M. T., and Swanson, J. A. (1996) *J. Cell Biol.* **135,** 1249–1260
46. He, Y., Hewlett, E., Temeles, D., and Quesenberry, P. (1988) *Blood* **71,** 1187–1195
47. Imamura, K., Dianoux, A., Nakamura, T., and Kufe, D. (1990) *EMBO J.* **9,** 2423–2389
48. Imamura, K., and Kufe, D. (1988) *J. Biol. Chem.* **263,** 14093–14098
49. Hume, D. A., and Denkins, Y. M. (1989) *Immunol. Cell Biol.* **67,** 243–249
50. Xie, Y., Von Gavel, S., Cassady, A. I., Stacey, K. J., Dunn, T. L., and Hume, D. A. (1993) *Br. J. Haematol.* **84,** 392–401
51. Corre, I., and Hermouet, S. (1995) *Blood* **86,** 1776–1783