

Activation of CR3-mediated phagocytosis by MSP requires the RON receptor, tyrosine kinase activity, phosphatidylinositol 3-kinase, and protein kinase C ζ

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Abstract: Macrophage-stimulating protein (MSP) promotes the phagocytosis of C3bi-coated erythrocytes by resident peritoneal macrophages, although the mechanism by which this occurs is largely unknown. We show that MSP-induced complement-mediated phagocytosis requires the RON receptor tyrosine kinase and the $\alpha M\beta 2$ integrin, as evidenced by the inability of RON $^{-/-}$ and $\alpha M^{-/-}$ peritoneal macrophages to augment phagocytosis of complement-coated sheep erythrocytes in response to MSP. MSP stimulation of macrophages results in tyrosine phosphorylation and AKT activation, and inhibitor studies demonstrate a phagocytic requirement for tyrosine kinase and phosphatidylinositol 3-kinase (PI-3K) activity as well as activity of the atypical protein kinase C (PKC) isoform ζ , which localizes to MSP-induced phagosomes containing complement-coated beads. Additionally, MSP augments the ability of peritoneal macrophages to bind to intercellular adhesion molecule-1 (ICAM-1) via the $\alpha M\beta 2$ integrin. MSP-induced ICAM-1 adhesion is also dependent on tyrosine kinase activity, PI-3K, and PKC ζ , indicating that these signaling requirements are upstream of complement receptor 3 activation. *J. Leukoc. Biol.* 73: 802–814; 2003.

Key Words: macrophage-stimulating protein · complement receptor 3 · intercellular adhesion molecule-1

INTRODUCTION

Phagocytosis of cellular debris by macrophages is an essential component of the innate-immune response. The mechanisms and consequences that govern this process can differ depending on the surface receptors that are responsible for the binding and ingestion of unwanted particles. Phagocytosis through the Fc receptor (Fc γ R) for immunoglobulin G (IgG) and complement receptor 3 (CR3; CD11b/CD18), which recognize IgG- and C3bi-opsonized targets, respectively, results in several differences in morphology, signaling requirements, and inflammatory responses. Specifically, Fc γ R-mediated phagocytosis is characterized by pseudopod extension, tyrosine kinase dependence, and the release of inflammatory mediators, and CR3-mediated phagocytosis is characterized by sinking of particles

into the cytoplasm, protein kinase C (PKC) dependence, and the absence of an inflammatory response [1]. The requirements for GTPases have also been shown to be different—CR3-mediated phagocytosis involves Rho and Rap1, and Fc γ R-mediated phagocytosis requires Rac and Cdc42 [2].

Additionally, Fc γ R-mediated phagocytosis can be initiated by clustering Ig receptors; however, CR3-mediated phagocytosis requires an additional signal, resulting in integrin activation. Resting macrophages can bind to C3bi-coated erythrocytes; however, rapid phagocytosis requires an additional signal. Signals for CR3 activation can be provided by a number of stimuli such as cytokines, chemokines, the presence of serum factors, attachment to the extracellular matrix (ECM), β -glucan binding, or stimulation with phorbol esters [3]. CR3 can be activated by “inside-out” or “outside-in” signaling mechanisms to generate high-affinity binding sites on the integrin. For example, lipopolysaccharide (LPS), formyl-Met-Leu-Phe (fMLP), or tumor necrosis factor α (TNF- α) can generate the high-affinity intercellular adhesion molecule-1 (ICAM-1)-binding state for CR3 [4]. Activation of CR3-mediated phagocytosis by the phorbol ester phorbol 12-myristate 13-acetate (PMA) occurs through the activation of PKC. Members of the PKC superfamily are characterized by serine/threonine kinase activity and can be divided into three subfamilies: classical, novel, and atypical. Activation of the classical isoforms (α , β I, β II, and γ) is Ca²⁺ and diacylglycerol (DAG)-dependent, and the novel isoforms (δ , ϵ , η , and θ) are DAG-dependent but Ca²⁺-independent. The atypical isoforms (ζ , λ/ι) are independent of Ca²⁺ and DAG [5].

Macrophage-stimulating protein (MSP) has also been shown to induce rapid phagocytosis of C3bi-coated erythrocytes via the CR3 receptor [6]. However, the signals required for this response are unknown. MSP is a serum protein, produced primarily by the liver as an inactive, 78-kDa pro-MSP, which requires proteolytic cleavage for activation to mature MSP. Pro-MSP is activated by members of the coagulation cascade including kallikrein, factor XIIa, or factor XIa [7] or by cell-surface pro-MSP convertase activity of peritoneal macrophages [8]. Activation of pro-MSP has been observed in wound exu-

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dates [9]. Mature MSP exerts several biological effects on resident peritoneal macrophages, which include induction of shape change, increased chemotaxis in response to C5a [10], down-regulation of nitric oxide (NO) production in response to cytokine and LPS stimulation [11–13], and induction of complement-mediated phagocytosis [6].

The biological effects of MSP are mediated through the RON receptor tyrosine kinase (RTK) [14] {murine stem cell kinase (STK) [15], chicken sarcoma, erythroblastosis, anemia (SEA) [16]}, which is highly homologous to the MET receptor. RON is not expressed on circulating monocytes, but its expression is up-regulated on populations of tissue-resident macrophages including resident peritoneal macrophages, Kupffer cells, and osteoclasts [17]. In addition, RON expression is down-regulated in response to LPS and proinflammatory cytokines through induction of NO production [11] but is up-regulated on day 3 peritoneal exudate macrophages and during tissue injury [18]. Mice with targeted deletions in RON are more susceptible to endotoxic shock and nickel-induced lung injury as well as exhibit increased inflammation in T helper cell type 1-mediated delayed-type hypersensitivity responses, all as a result of, at least in part, increased NO production by activated macrophages [19–21]. In addition, RON-deficient mice are resistant to Friend virus (FV)-induced erythroleukemia, confirming the identity of the STK/RON receptor as the FV susceptibility-2 locus in mice [22].

Activation of the RON receptor by MSP can lead to the activation of multiple signaling pathways, including the phosphatidylinositol 3-kinase (PI-3K) and mitogen-activated protein kinase (MAPK) cascades, through phosphorylation of a two tyrosine-docking site in the C-terminal tail [23]. The ability of MSP to down-regulate NO production and inducible NO synthase (iNOS) expression in response to cytokine and LPS stimulation is dependent on RON-mediated activation of the PI-3K pathway [13]. PI-3K is also involved in MSP-induced, integrin-mediated epithelial cell adhesion to collagen or fibronectin [24]. Integrin activation by RTKs has been extensively studied. Although the mechanism by which RTKs activate integrins is not entirely understood, activation of PI-3K, PKC, and the rho family of GTPases has been implicated. This paper demonstrates that MSP-induced activation of CR3-mediated phagocytosis and ICAM-1 binding is dependent on the RON RTK and the downstream signaling molecules PI-3K and PKC ζ .

MATERIALS AND METHODS

Mice

RON^{-/-} mice on a CD-1 background were generated as described previously [21], and their littermates were used as controls. CR3 null mice ($\alpha M^{-/-}$) on the C57BL/6 strain were used, and wild-type C57BL/6 mice were used as controls. For the inhibitor studies, BALB/c mice were used.

Isolation of peritoneal macrophages

Resident peritoneal macrophages were harvested by washing the peritoneal cavity with 5 ml Dulbecco's modified Eagle's medium (DMEM). The cells were then plated onto 16-well chamber slides at 1×10^4 cells/well. After 1 h,

nonadherent cells were removed by washing the slides three times with DMEM.

Opsonization of sheep erythrocytes and latex beads

Sheep erythrocytes, collected in alsevers (Hemostat, Dixon, CA) were washed twice in versicolorin B synthase (VBS) gel (Sigma Chemical Co., St. Louis, MO). For C3bi opsonization, erythrocytes were incubated with a subagglutinating titer of IgM antish sheep erythrocyte antibody (Serotec, Oxford, UK) for 1 h followed by 30 min incubation with C5-deficient serum (Sigma Chemical Co.) at 37°C. For IgG opsonization, erythrocytes were incubated with a subagglutinating titer of IgG antish sheep erythrocyte antibody (Sigma Chemical Co.) for 1 h at 37°C. Opsonized erythrocytes were washed three times in VBS gel and resuspended in DMEM. For complement opsonization of latex beads, carboxylated 4.5 μ latex beads (Polysciences, Inc., Warrington, PA) were incubated for 1 h at 37°C with fresh mouse serum and then washed 3x with Hanks' balanced salt solution (Sigma Chemical Co.) before being resuspended in DMEM.

Phagocytosis assay

For phagocytosis, opsonized erythrocytes were added to the macrophages at a final concentration of 5×10^5 erythrocytes/well for 5 min to allow for binding. Complement-mediated phagocytosis was then initiated with the addition of 100 ng/ml MSP (R&D Systems, Minneapolis, MN) or the indicated dose of PMA (Sigma Chemical Co.). After allowing phagocytosis to proceed at 37°C for 1 h, the chamber slides were washed twice with phosphate-buffered saline (PBS), and ammonium chloride lysis buffer (0.16 M NH₄Cl, 0.01 M KHCO₃, 0.001 M EDTA, pH 7.4) was added for 2 min. Slides were then stained with Diff-Quik (Dade Behring, Switzerland). The gasket was removed, and coverslips were mounted for examination under 100 \times oil immersion objective. Percent phagocytosis was determined by expressing the number of macrophages to engulf at least one erythrocyte out of the total number of at least 200 macrophages counted. Phagocytic index was determined by counting the total number of phagocytic events per 100 macrophages. Binding was determined by using a modified hemolytic assay and visual inspection. Briefly, varying concentrations of erythrocytes were allowed to adhere to macrophages for 15 min, and unbound erythrocytes were removed by washing three times with PBS. Bound erythrocytes were lysed using H₂O, read on a plate reader at 540 nm, and binding was determined by calibrating values against a standard curve.

Inhibitors

The biochemical inhibitors wortmannin, LY294002, bisindolylmaleimide I, bisindolylmaleimide V, PD98059, SB203580, cytochalasin B, and herbimycin A (Calbiochem, San Diego, CA) and chelerythrine chloride (Alexis Biochemicals, San Diego, CA) were used in these studies. Macrophages were pretreated with wortmannin or LY294002 for 1 h; PPI, bisindolylmaleimide I, bisindolylmaleimide V, chelerythrine chloride, PD98059, or SB203580, for 30 min; or herbimycin A, for 4 h before the induction of phagocytosis at the indicated concentrations of each inhibitor. Peritoneal macrophages stimulated with 2 μ g/ml PMA for 24 h were washed twice before induction of phagocytosis. After pretreatment for 15 min using the myristoylated ζ pseudosubstrate peptide, SIYRRGARRWRKL, or the myristoylated θ pseudosubstrate peptide, LHQRGAIKQAKVHHVQC (Biosource International, Camarillo, CA), the cells were washed twice before induction of phagocytosis. The biochemical inhibitors and PMA were dissolved in dimethylsulfoxide (DMSO), the myristoylated peptides were dissolved at 37°C in DMEM, and the amount of inhibitor or control did not exceed 0.1% of total volume when used. Percent phagocytosis is expressed as the percent phagocytosis in the presence of inhibitor divided by control and was determined for each inhibitor relative to the respective controls (DMSO or DMEM).

Western blotting

Plates (100 mm) of peritoneal macrophages were washed with ice-cold PBS and lysed using 1 ml lysis buffer (1% digitonin, 150 mM NaCl, 0.4 mM EDTA, pH 7.4, 10 mM NaF, 2 mM Na₃VO₄, and 2 mM phenylmethylsulfonyl fluoride) on ice for 15 min. The lysates were cleared by centrifugation at 12,000 rpm for 15 min at 4°C. The protein concentration was measured using the DC protein assay kit (Bio-Rad, Richmond, CA) and a Beckman DU530 spectrophotometer

(Palo Alto, CA). Equal amounts of protein were resuspended in $2.5 \times$ sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE; 10%). The gels were transferred to a nitrocellulose membrane, which was blocked in 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h and were incubated with antiphosphotyrosine (Upstate Biotechnology, Lake Placid, NY) or the indicated PKC antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was washed extensively with TBST and incubated with peroxidase-conjugated secondary antibodies. Proteins were visualized by SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and X-ray film.

AKT kinase assay

This assay was performed using a nonradioactive inositol phosphatase (IP) kinase assay kit (Cell Signaling Technology, Beverly, MA). Briefly, equal protein amounts of peritoneal cell lysates were immunoprecipitated using immobilized AKT antibody beads. IP pellets were resuspended in kinase buffer and incubated with adenosine 5'-triphosphate and glycogen synthase kinase 3 (GSK-3) fusion protein for 30 min at 30°C. By adding SDS sample buffer, boiled and separated by SDS-PAGE, the reaction was terminated. Nitrocellulose membranes were blotted with a phospho-GSK-3 α/β (Ser21/9) antibody and were detected as previously mentioned. Equal amounts of lysates used in the AKT kinase assay were analyzed by Western blot using a rabbit polyclonal AKT antibody (Cell Signaling Technology) to show equivalent protein levels.

ICAM-1 binding and flow cytometric analysis

To detect ICAM-1 binding, peritoneal macrophages were harvested, washed with DMEM, and resuspended at 1×10^7 cells/ml. Macrophages were pretreated with or without inhibitor before the addition of stimulus and $1 \mu\text{g/ml}$ mouse ICAM-1/human Fc chimera protein (R&D Systems) for 30 min. Cells were spun down and resuspended in PBS/2% newborn calf serum (NCS) on ice with $10 \mu\text{g/ml}$ fluorescein isothiocyanate (FITC)-conjugated human Fc-specific antibody (Sigma Chemical Co.) for 60 min. Cells were washed three times in PBS/2% NCS, resuspended in 1 ml PBS/2% NCS with 10 mg/ml propidium iodide, and analyzed by flow cytometry (XL; Coulter, Hialeah, FL). To detect the expression of CD11b, peritoneal macrophages were similarly prepared and incubated with $1 \mu\text{l}$ FITC-labeled anti-integrin α_M or FITC-labeled isotype control-matched anti-IgG κ (PharMingen, San Diego, CA).

Immunofluorescence

Residential peritoneal macrophages were plated onto glass coverslips. Macrophages were stimulated with MSP or PMA in the presence of complement-coated latex beads. After 1 h, the cells were washed in PBS before fixation with ice-cold methanol for 20 min at -20°C . Coverslips were blocked with 1% bovine serum albumin/PBS before 1 h labeling with $10 \mu\text{g/ml}$ rabbit polyclonal IgG anti-PKC ζ , $10 \mu\text{g/ml}$ rabbit polyclonal IgG anti-PKC δ , polyclonal IgG anti-PKC ϵ , or $10 \mu\text{g/ml}$ control IgG (Santa Cruz Biotechnology). Cells were then washed with PBS 3x and incubated with a 1:100 dilution of secondary FITC-labeled antirabbit IgG (Santa Cruz Biotechnology). Coverslips were then washed 3x in PBS and mounted on microslides with 50% glycerol/PBS. For actin visualization, samples were prepared as described above except fixed with 3.7% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100/PBS, and labeled with 5 units/ml Alexa Fluor 568 phalloidin (Molecular Probes, Eugene, OR). Immunofluorescence was analyzed using an Olympus confocal laser-scanning microscope (Olympus America Inc., Melville, NY).

RESULTS

MSP-induced activation of complement-mediated phagocytosis requires the RON RTK and CR3

To determine whether MSP-induced activation of complement-mediated phagocytosis occurs through the RON receptor, peritoneal macrophages from RON +/+, +/-, and -/- mice were

assayed for their ability to engulf C3bi- and IgG-opsonized sheep erythrocytes in the presence or absence of MSP. As shown in **Figure 1A**, phagocytosis of C3bi-coated targets does not occur in the absence of MSP regardless of genotype. However, upon addition of MSP, only wild-type macrophages were able to efficiently ingest C3bi-coated erythrocytes, and the RON -/- macrophages were unable to ingest C3bi-coated erythrocytes. Macrophages from mice heterozygous for the targeted allele showed an intermediate response. No significant differences in phagocytosis of IgG-coated erythrocytes were observed among RON +/+, +/-, and -/- macrophages stimulated with or without MSP (Fig. 1B).

To determine whether RON -/- macrophages were deficient in CR3-mediated phagocytosis in response to stimuli other than MSP, activation of CR3-mediated phagocytosis by PMA was compared in RON +/+ and -/- macrophages. As seen in **Figure 1C**, wild-type and RON -/- peritoneal macrophages were able to phagocytose C3bi-opsonized targets in response to PMA in a dose-dependent manner. Therefore, PMA induction of complement-mediated phagocytosis is independent of the RON receptor.

To confirm whether MSP-induced activation of complement-mediated phagocytosis occurs through the $\alpha\text{M}\beta 2$ integrin, peritoneal macrophages from $\alpha\text{M} +/+$ and -/- mice were assayed for their ability to engulf C3bi-coated erythrocytes in the presence or absence of MSP. As seen in **Figure 1D**, phagocytosis of C3bi-coated targets does not occur in the absence of MSP regardless of genotype. However, upon addition of MSP, only wild-type macrophages were able to efficiently ingest C3bi-coated erythrocytes, and the $\alpha\text{M} -/-$ macrophages were unable to ingest C3bi-coated erythrocytes. Therefore, MSP-induced phagocytosis is occurring through the CR3 receptor.

MSP stimulation of the RON receptor results in the activation of CR3 as measured by binding to ICAM-1

To ascertain whether MSP stimulation causes changes in surface levels of CR3, expression of CR3 was analyzed by flow cytometry. As shown in **Figure 2A**, MSP stimulation does not change the levels of CD11b expression on peritoneal macrophages, suggesting that MSP-induced phagocytosis is not a result of changes in CR3 expression. Additionally, MSP stimulation did not change the ability of macrophages to bind to varying concentrations of C3bi-coated erythrocytes, and the binding of C3bi-coated erythrocytes was independent of the RON receptor (Fig. 2B).

To determine whether MSP stimulation results in the activation of CR3 by inducing the high-affinity state for ICAM-1, the ability of peritoneal macrophages to bind to ICAM-1 was measured. MSP and PMA were able to induce increased ICAM-1 binding in RON +/+ macrophages (Fig. 2C); however, only PMA was able to induce increased ICAM-1 binding in RON -/- macrophages (Fig. 2D). Therefore, the ability of MSP to augment ICAM-1 binding is dependent on the RON receptor. Additionally, the ability of macrophages to bind to ICAM-1 is dependent on the αM subunit of CR3, as evidenced

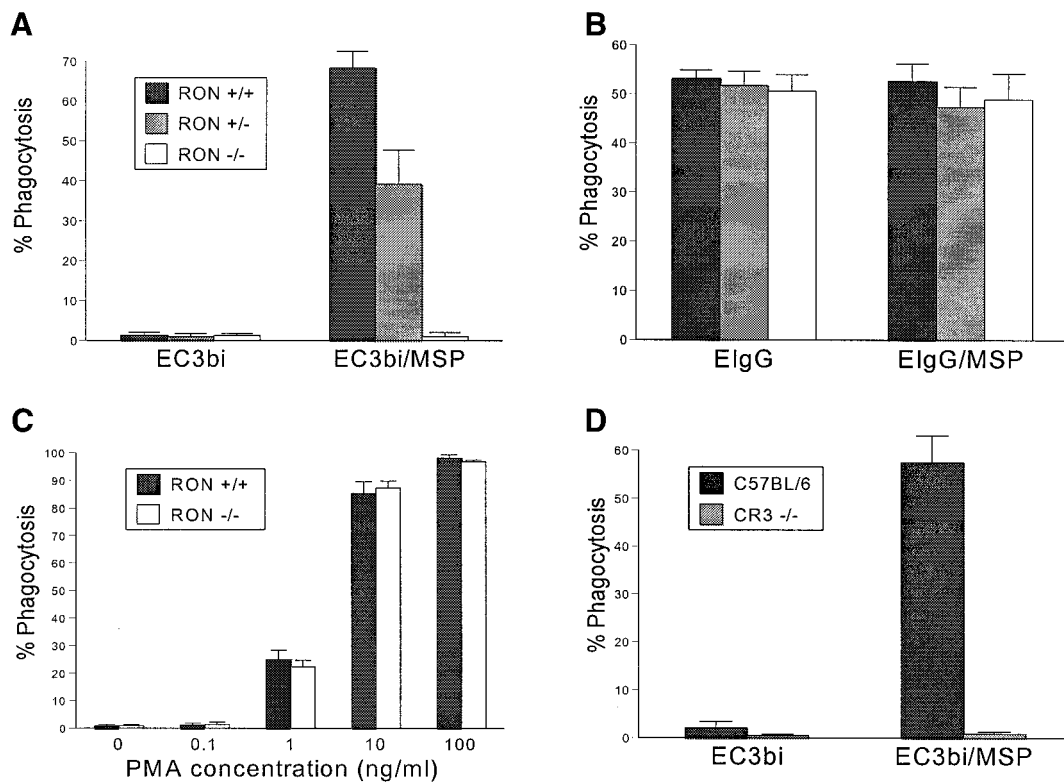


Fig. 1. RON^{-/-} and α M null peritoneal macrophages fail to engulf C3bi-opsonized sheep erythrocytes in response to MSP. (A) Phagocytosis of complement-coated erythrocytes (EC3bi) or of (B) Ig-coated erythrocytes (IgG) was assayed in peritoneal macrophages harvested from RON^{+/+}, ^{+/-}, and ^{-/-} mice in the presence or absence of 100 ng/ml MSP. (C) Phagocytosis of C3bi-opsonized erythrocytes by peritoneal macrophages harvested from RON^{+/+}, ^{+/-}, and ^{-/-} mice was measured in the presence of indicated doses of PMA. (D) Phagocytosis of complement-coated erythrocytes was assayed in peritoneal macrophages harvested from CR3^{-/-} mice and their C57BL/6 counterparts in the presence or absence of 100 ng/ml MSP.

by decreased binding of ICAM-1 in response to PMA by macrophages from α M^{-/-} mice when compared with α M^{+/-} controls (Fig. 2E).

MSP-induced activation of CR3-mediated phagocytosis requires tyrosine kinase activity

To determine whether tyrosine kinase activity is required in CR3-mediated phagocytosis induced by MSP, we used the potent tyrosine kinase inhibitor herbimycin A. MSP stimulation results in the phosphorylation of tyrosine residues in primary peritoneal macrophages from wild-type mice (Fig. 3A) but not macrophages from RON^{-/-} mice (Fig. 3B). Figure 3B also demonstrates that the tyrosine kinase inhibitor can block tyrosine phosphorylation in response to MSP. Inhibitor studies using herbimycin A support a requirement for tyrosine kinase activity in MSP-induced, CR3-mediated phagocytosis, as demonstrated by the dose-dependent blockade of phagocytosis in response to MSP in the presence of herbimycin A. Conversely, activation of CR3-mediated phagocytosis by PMA was not inhibited by herbimycin A, indicating tyrosine kinase activity is dispensable for this route of induction (Fig. 3C). Herbimycin A was also able to inhibit MSP-induced ICAM-1 binding but not PMA-induced ICAM-1 binding (Fig. 3D). Pretreatment of these cells with herbimycin A or any of the subsequent inhibitors used in this paper did not significantly alter CD11b expression or the ability of C3bi-coated erythrocytes to bind to resting macrophages as shown in Table 1.

MSP-induced activation of CR3-mediated phagocytosis requires PI-3K activity

PI-3K has been shown to mediate several of the activities induced by MSP/RON [23]. Therefore, the ability of MSP to activate AKT, a downstream effector of PI-3K, in primary peritoneal macrophages was analyzed using an in vitro kinase assay. As demonstrated in Figure 4A, MSP stimulation increases AKT activity as measured by increased phosphorylation of the AKT substrate GSK-3 α/β over time. The two structurally unrelated inhibitors wortmannin and LY294002 were used to selectively inhibit PI-3K activity. Here, we show that wortmannin and LY294002 block MSP-induced AKT activation (Fig. 4B). Herbimycin A was also able to block AKT activation by MSP in RON^{+/+} macrophages, indicating that tyrosine phosphorylation is upstream of PI-3K activity following MSP stimulation. Additionally, PI-3K-dependent AKT activation requires the RON receptor, as RON^{-/-} macrophages were not responsive to MSP-induced AKT activation (Fig. 4C).

The ability of macrophages to ingest C3bi-coated erythrocytes in response to MSP was examined in the presence of wortmannin and LY294002. Pretreatment of peritoneal macrophages with wortmannin (Fig. 5A) or LY294002 (Fig. 5B) was able to block MSP-induced activation of CR3-mediated phagocytosis in a dose-dependent manner, indicating that PI-3K is required for MSP-induced complement-mediated phagocytosis. Alternatively, wortmannin (Fig. 5A) and LY294002 (Fig. 5B)

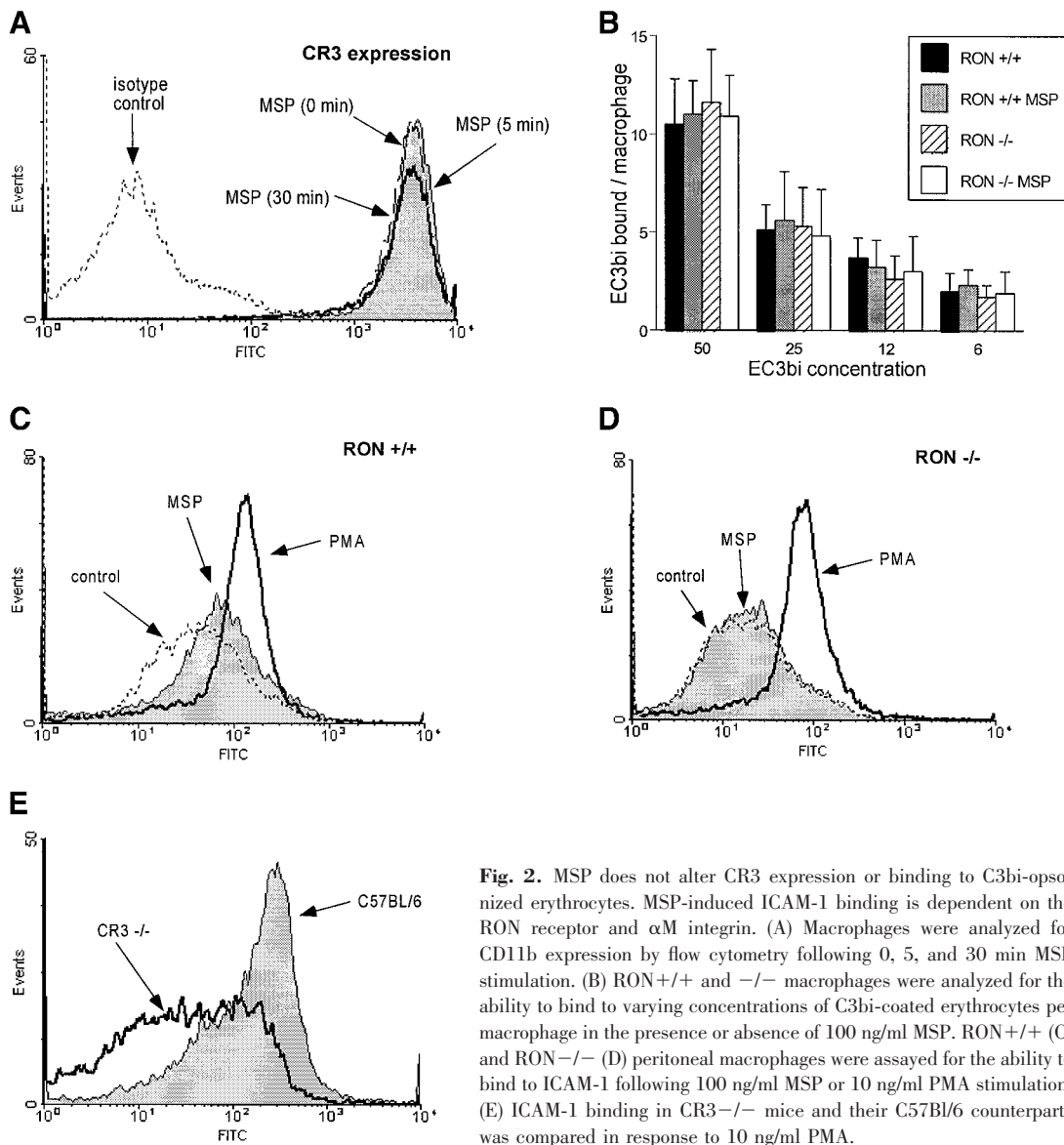


Fig. 2. MSP does not alter CR3 expression or binding to C3bi-opsonized erythrocytes. MSP-induced ICAM-1 binding is dependent on the RON receptor and α M integrin. (A) Macrophages were analyzed for CD11b expression by flow cytometry following 0, 5, and 30 min MSP stimulation. (B) RON^{+/+} and ^{-/-} macrophages were analyzed for the ability to bind to varying concentrations of C3bi-coated erythrocytes per macrophage in the presence or absence of 100 ng/ml MSP. RON^{+/+} (C) and RON^{-/-} (D) peritoneal macrophages were assayed for the ability to bind to ICAM-1 following 100 ng/ml MSP or 10 ng/ml PMA stimulation. (E) ICAM-1 binding in CR3^{-/-} mice and their C57BL/6 counterparts was compared in response to 10 ng/ml PMA.

were unable to inhibit PMA-induced CR3-mediated phagocytosis. We cannot, however, rule out PI-3K as a requirement for PMA-induced, complement-mediated phagocytosis, as wortmannin was unable to block PMA-induced AKT activation at the dose used for these studies (data not shown). Similarly, wortmannin (Fig. 5C) and LY294002 (Fig. 5D) were able to block MSP-induced ICAM-1 binding but not PMA-induced ICAM-1 binding, demonstrating a requirement for PI-3K in the activation of CR3 by MSP.

To determine whether MAPK activation downstream of the RON receptor is required for MSP- and PMA-induced, CR3-mediated phagocytosis, the ability of peritoneal macrophages to ingest C3bi-coated erythrocytes in response to MSP or PMA in the presence of the chemical inhibitors PD98059 and SB203580, which selectively inhibit MAPK kinase (MEK)1 and p38, respectively, was determined. At the effective range for inhibition of MEK1 and p38, PD98059 and SB20350 did not have any significant effect on phagocytosis, suggesting that MEK1 and p38 are not required for MSP- or PMA-induced, CR3-mediated phagocytosis (data not shown).

MSP-induced, CR3-mediated phagocytosis requires DAG-independent PKC activation

A role for PKC in PMA-induced, CR3-mediated phagocytosis has been established previously [25]. Therefore, the uptake of C3bi-coated erythrocytes in response to MSP in the presence of PKC inhibitors was examined and compared with PMA-induced, CR3-mediated phagocytosis. Chelerythrine chloride, which inhibits all isoforms of PKC in a nonselective manner, inhibited MSP- and PMA-induced, complement-mediated phagocytosis at the effective dose (Fig. 6A). To pinpoint which isoform(s) were responsible for phagocytosis, the PKC inhibitor bisindolylmaleimide I and the related inactive analog bisindolylmaleimide V were used. The classical PKC isoforms and the novel PKC isoforms are more sensitive to inhibition by bisindolylmaleimide I compared with the atypical isoforms. Bisindolylmaleimide I (Fig. 6B) but not bisindolylmaleimide V (Fig. 6C) resulted in a differential inhibition curve for CR3-mediated phagocytosis induced by MSP and PMA, and substantial inhibi-

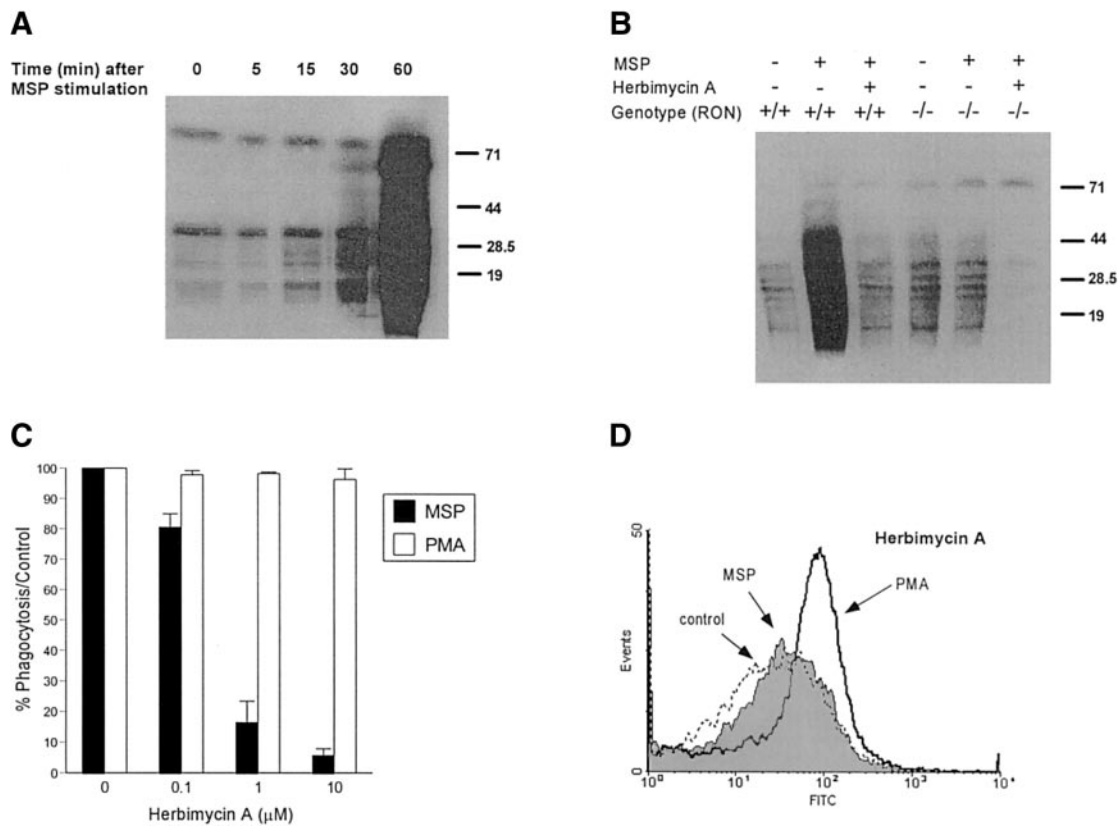


Fig. 3. MSP induces tyrosine phosphorylation in RON wild-type peritoneal macrophages but not in RON knockout mice. The tyrosine kinase inhibitor, herbimycin A, blocks MSP tyrosine phosphorylation and subsequent MSP-induced, CR3-mediated phagocytosis and MSP-induced ICAM-1 binding. (A) Protein lysates from resident peritoneal macrophages were harvested at various times after stimulation with 100 ng/ml MSP. Equal amount of protein lysates were separated by SDS-PAGE, and phosphotyrosine residues were detected by Western blot. (B) Protein lysates from resident peritoneal macrophages from RON^{+/+} and RON^{-/-} mice were pretreated with DMSO control or 10 μ M herbimycin A for 4 h before stimulation with 100 ng/ml MSP. Equal amount of protein lysates were separated by SDS-PAGE, and phosphotyrosine residues were detected by Western blot. (C) Resident peritoneal macrophages were pretreated with different doses of herbimycin A for 4 h before the phagocytosis of C3bi-opsonized erythrocytes in response to 100 ng/ml MSP or 10 ng/ml PMA. (D) Peritoneal macrophages were analyzed by flow cytometry for ICAM-1 binding following 100 ng/ml MSP or 10 ng/ml PMA stimulation in the presence of herbimycin A.

tion of MSP-induced phagocytosis occurred only at higher doses. High doses of bisindolylmaleimide I also inhibited the ability of MSP and PMA to induce ICAM-1 binding to peritoneal macrophages (Fig. 6D). However, at these higher doses, bisindolylma-

leimide I becomes less selective. Therefore, other methods for examining atypical PKCs were used.

Prolonged exposure to high doses of PMA can down-regulate the classical and novel PKC isoforms with little effect on the

TABLE 1. Effect of Drug Treatments on CD11b Expression, Phagocytic Index, and Binding of C3bi-Coated Erythrocytes to Peritoneal Macrophages

Treatment	CD11b Mean fluorescence	Phagocytic index	EC3bi binding
Untreated	590.1	124.0 \pm 10.4	9.5 \pm 2.5
DMSO (0.1 %)	560.9	112.1 \pm 17.1	12.9 \pm 5.7
Herbimycin A (10 μ M)	516.6	7.0 \pm 3.0	10.0 \pm 1.2
Wortmannin (100 nM)	464.5	1.7 \pm 1.2	10.5 \pm 4.0
LY294002 (10 μ M)	533.1	1.3 \pm 0.6	6.5 \pm 3.1
PD98059 (25 μ M)	567.6	104 \pm 26.9	14.9 \pm 4.8
SB203580 (10 μ M)	550	89.5 \pm 16.3	13.0 \pm 4.0
Bisindolylmaleimide I (20 μ M)	494.6	23.0 \pm 0.6	11.5 \pm 5.3
Bisindolylmaleimide V (20 μ M)	ND	116 \pm 4.2	9.7 \pm 0.3
Chelerythrine chloride (10 μ M)	348.3	2.3 \pm 1.5	11.2 \pm 5.6
PKC ζ pseudosubstrate (50 μ M)	540.5	1.5 \pm 0.7	8.1 \pm 0.8
PKC θ pseudosubstrate (50 μ M)	450.5	142.5 \pm 14.8	12.1 \pm 7.6
Cytochalasin B (10 μ M)	584.2	3 \pm 1.4	14.5 \pm 3.6

Peritoneal macrophages were pretreated with the indicated inhibitors and then analyzed for CD11b expression by flow cytometry. In addition to percent-phagocytosis, phagocytic indexes were scored as total number of engulfed erythrocytes per 100 macrophages, and the number of bound erythrocytes per macrophage was determined.

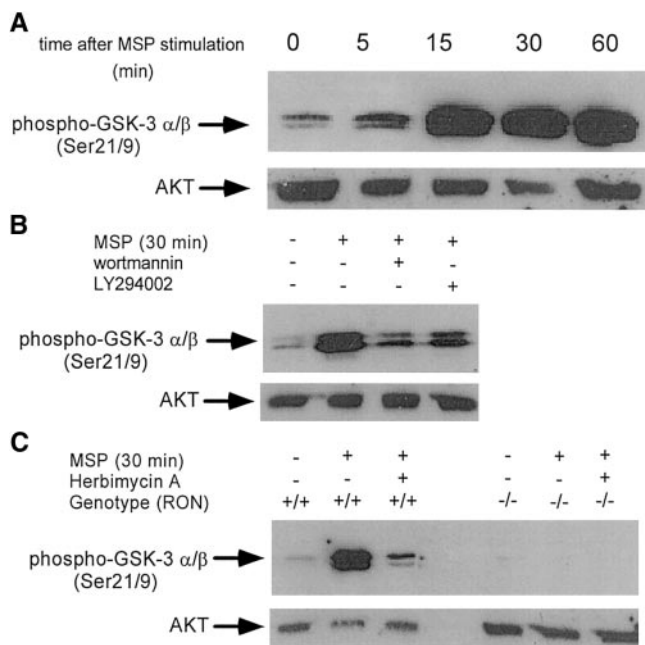


Fig. 4. MSP stimulation induces tyrosine PI-3K-dependent AKT activation in peritoneal macrophages. (A) Protein lysates from peritoneal macrophages stimulated with 100 ng/ml MSP for various times were subjected to an *in vitro* AKT kinase assay. (B) Protein lysates from peritoneal macrophages pretreated for 1 h with wortmannin (100 nM) and LY294002 (10 μ M) before stimulation with 100 ng/ml MSP for 30 min were subjected to an *in vitro* AKT kinase assay (C). Protein lysates from peritoneal macrophages from RON+/+ or -/- mice were pretreated for 4 h with herbimycin A (10 μ M), before stimulation with 100 ng/ml MSP for 30 min was subjected to an *in vitro* AKT kinase assay.

atypical PKC isoforms. Figure 6E shows PKC isoform expression in peritoneal macrophages that were pretreated with high-dose PMA over time. By 16 h, the novel isoforms δ and ϵ are down-regulated, and the ζ isoform is unaffected. Following prolonged PMA pretreatment, peritoneal macrophages were still responsive to induction of phagocytosis by MSP but not to PMA (Fig. 6F), suggesting that MSP-induced, CR3-mediated phagocytosis does not require the activation of DAG-dependent PKCs. Taken together, these data suggest a potential role for involvement of atypical PKC isoforms in MSP-induced, CR3-mediated phagocytosis.

CR3-mediated phagocytosis induced by MSP requires PKC ζ activity and F-actin reorganization

PI-3K has been shown to play a role in the activation of PKC ζ through the activation of phosphoinositide-dependent protein kinase-1 (PDK-1) [26]. To determine whether PKC ζ plays a role in MSP-induced, complement-mediated phagocytosis, the myristoylated pseudosubstrate peptide for PKC ζ was used. As a control, the myristoylated PKC θ pseudosubstrate peptide was used to demonstrate that the presence of the myristoylated peptides did not nonspecifically inhibit phagocytosis. The PKC ζ pseudopeptide inhibited MSP-induced, complement-mediated phagocytosis in a dose-dependent manner, and the control PKC θ pseudopeptide had no effect (Fig. 7A). Similar results were shown for PMA-induced, complement-mediated phagocytosis (Fig. 7B).

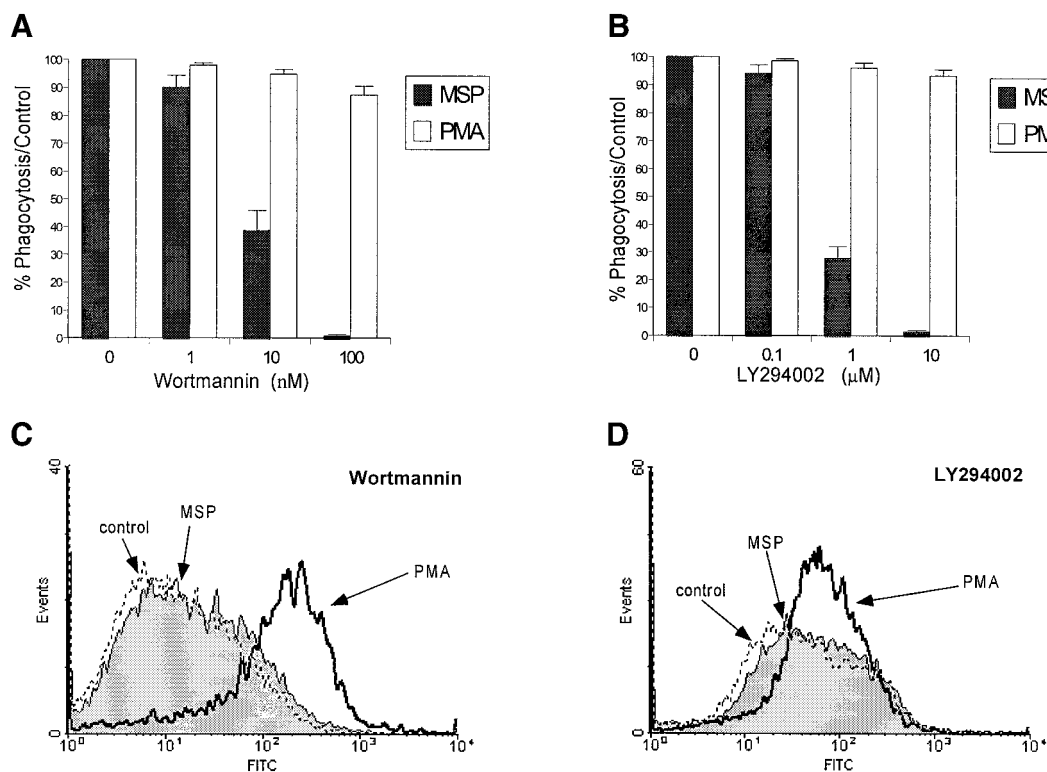


Fig. 5. The PI-3K inhibitors wortmannin and LY294002 block MSP-induced activation of CR3-mediated phagocytosis and MSP-induced ICAM-1 binding. Resident peritoneal macrophages were pretreated with the indicated doses of wortmannin (A) or LY294002 (B) for 1 h before the phagocytosis of C3bi-opsonized erythrocytes in response to 100 ng/ml MSP or 10 ng/ml PMA. Peritoneal macrophages were assayed by flow cytometry for ICAM-1 binding following 100 ng/ml MSP or 10 ng/ml PMA stimulation in the presence of wortmannin (C) or LY294002 (D).

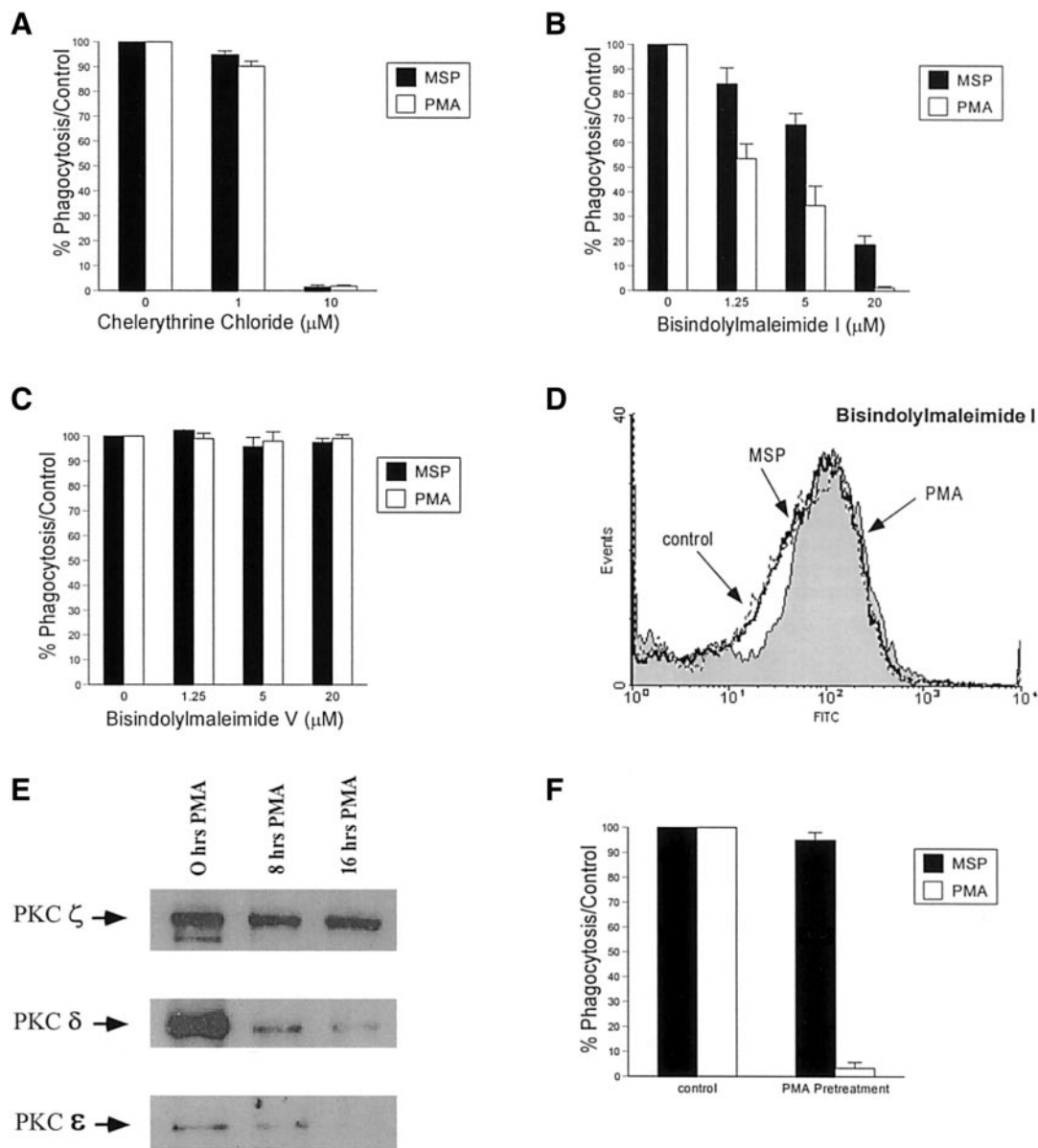


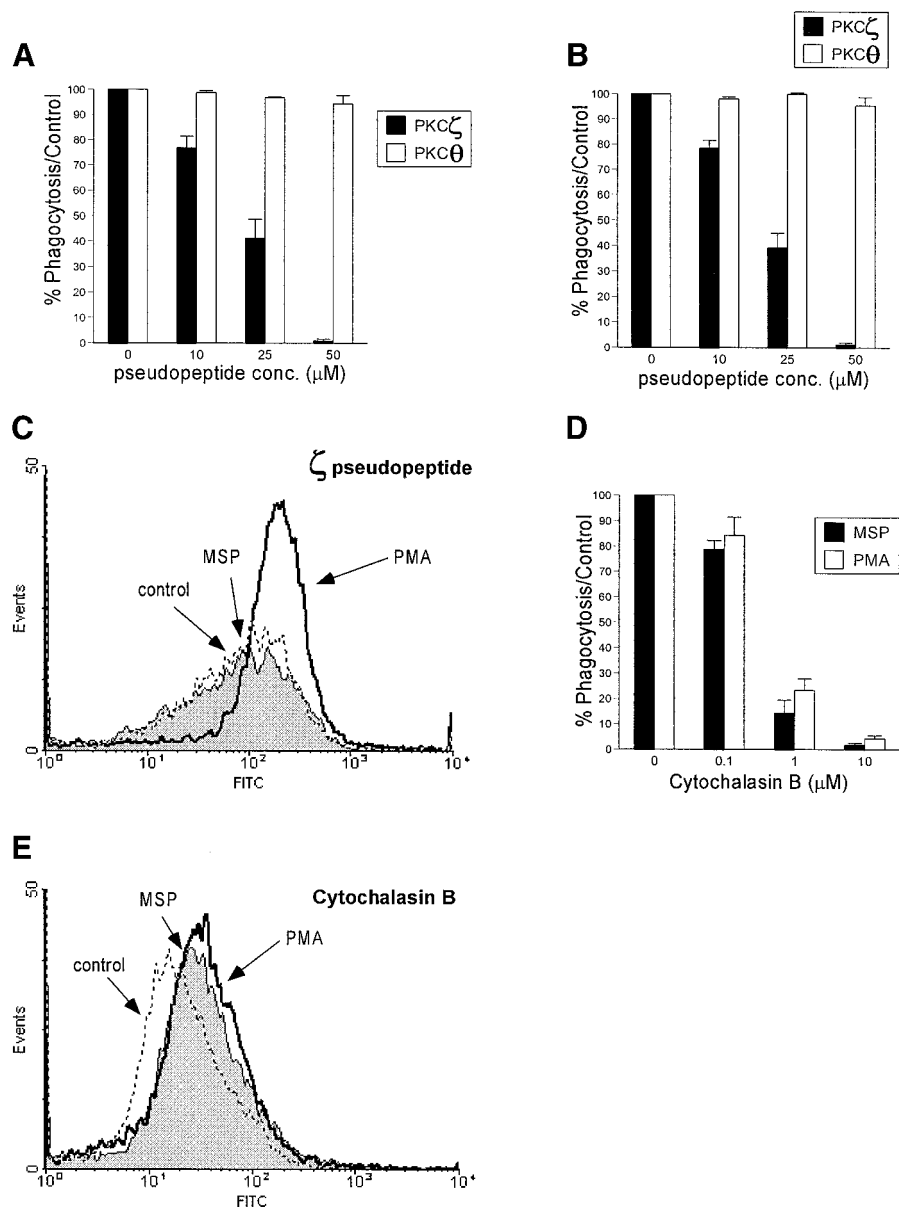
Fig. 6. The biochemical PKC inhibitors bisindolylmaleimide I and chelerythrine chloride inhibit MSP-induced, CR3-mediated phagocytosis differentially, and prolonged, high-dose PMA pretreatment does not inhibit MSP-induced, CR3-mediated phagocytosis. Resident peritoneal macrophages were pretreated with various doses of chelerythrine chloride (A), bisindolylmaleimide I (B), or bisindolylmaleimide V (C) for 30 min before induction of complement-mediated phagocytosis with 100 ng/ml MSP or 10 ng/ml PMA. (D) Peritoneal macrophages were analyzed by flow cytometry for ICAM-1 binding following 100 ng/ml MSP or 10 ng/ml PMA stimulation in the presence of bisindolylmaleimide I. (E) Expression of PKC isoforms in residential peritoneal macrophages was analyzed by Western blot following high-dose PMA (2 $\mu\text{g/ml}$) exposure. (F) Resident peritoneal macrophages were pretreated with 2 $\mu\text{g/ml}$ PMA for 24 h before induction of complement-mediated phagocytosis with 100 ng/ml MSP or 10 ng/ml PMA.

The PKC ζ pseudosubstrate was also able to inhibit MSP-induced ICAM-1 binding (Fig. 7C), which would indicate that PKC ζ is required for the activation of the CR3 receptor in response to MSP. However, the PKC ζ pseudosubstrate was unable to inhibit PMA-induced ICAM-1 binding (Fig. 7C), suggesting that PMA can activate other PKC isoforms that could compensate for PKC ζ in the activation of CR3. As expected, the control PKC θ pseudosubstrate was unable to inhibit MSP- or PMA-induced ICAM-1 binding (data not shown), demonstrating that the inhibition was specific to the PKC ζ pseudosubstrate. Additionally, PKC ζ and PKC θ pseudosubstrates were unable to inhibit zymosan-induced

ICAM-1 binding (data not shown), indicating that the PKC ζ pseudosubstrate is specific to blocking inside-out integrin activation. Although PKC ζ may share common substrates with other isoforms, such as PKC ϵ , the pseudosubstrate data in combination with results from the PMA pretreatment suggest that PKC ζ activity is required for MSP-induced CR3 activation and complement-mediated phagocytosis.

F-actin reorganization has been shown to be an integral part of IgG- and complement-mediated phagocytosis [27]. In addition, MSP has been shown to induce F-actin reorganization in BaF/3 cells transfected with the wild-type RON receptor [28]. Therefore, we set out to determine whether F-actin reorgani-

Fig. 7. MSP-induced, CR3-mediated phagocytosis is inhibited by the specific PKC ζ pseudopeptide inhibitor but not the specific PKC θ pseudopeptide inhibitor, and CR3-mediated phagocytosis induced by MSP is inhibited by the cytoskeletal inhibitor cytochalasin B. Peritoneal macrophages were pretreated with the indicated doses of myristoylated ζ pseudosubstrate peptide, SIYRRGARRWRKL, or control myristoylated θ pseudosubstrate peptide, LHQRRGAIKQAKVHHVQC, for 30 min before induction of complement-mediated phagocytosis by 100 ng/ml MSP (A) or 10 ng/ml PMA (B). (C) Peritoneal macrophages were assayed by flow cytometry for ICAM-1 binding following 100 ng/ml MSP or 10 ng/ml PMA stimulation in the presence of the PKC ζ pseudosubstrate peptide. (D) Peritoneal macrophages were pretreated with varying doses of cytochalasin B for 30 min before induction of phagocytosis by 100 ng/ml MSP. (E) Peritoneal macrophages were analyzed by flow cytometry for ICAM-1 binding following 100 ng/ml MSP or 10 ng/ml PMA stimulation in the presence of cytochalasin B.



zation is required for MSP-induced ingestion of complement-coated particles. Consistent with previous studies, our results suggest that MSP-induced, CR3-mediated phagocytosis is dependent on F-actin reorganization, as evidenced by the ability of cytochalasin B to inhibit phagocytosis by MSP in a dose-dependent manner (Fig. 7D). Figure 7D also shows similar results for cytochalasin B exhibiting dose-dependent inhibition of PMA-induced, complement-mediated phagocytosis. Alternatively, F-actin is dispensable for MSP- and PMA-induced ICAM-1 binding, as evidenced by the inability of cytochalasin B to block this process (Fig. 7E).

Our data indicate that in addition to a role in activating CR3 in response to MSP, PKC ζ is also important downstream of the integrin, as evidenced by the ability of the PKC ζ pseudosubstrate to block MSP- and PMA-induced phagocytosis (Fig. 7A). To examine the localization of PKC ζ , macrophages treated with MSP in the presence of complement-coated latex beads were stained with a FITC-labeled anti-PKC ζ antibody. Con-

focal immunofluorescence microscopy demonstrated that PKC ζ (Fig. 8A) localizes to MSP- and PMA-induced phagosomes containing complement-coated latex beads. However, control IgG antibodies (Fig. 8B) and PKC δ antibodies (Fig. 8C) did not localize to the phagosomes under these conditions. Additionally, the F-actin stain, phalloidin, accumulates around MSP- and PMA-induced phagosomes (Fig. 8D). It is interesting that PKC ϵ was also observed to localize to MSP- and PMA-induced phagosomes (data not shown). Similar observations were seen using complement-coated sheep erythrocytes showing colocalization of PKC ζ and F-actin to MSP- and PMA-induced phagosomes by confocal immunofluorescence microscopy (data not shown). These data also strengthen the argument for the role of PKC ζ in the downstream cytoskeletal events involved in MSP-induced, CR3-mediated phagocytosis. **Figure 9** summarizes the role of PKC ζ as well as other signaling requirements in relationship to the RON receptor and CR3 for MSP-induced phagocytosis and MSP-induced ICAM-1 binding.

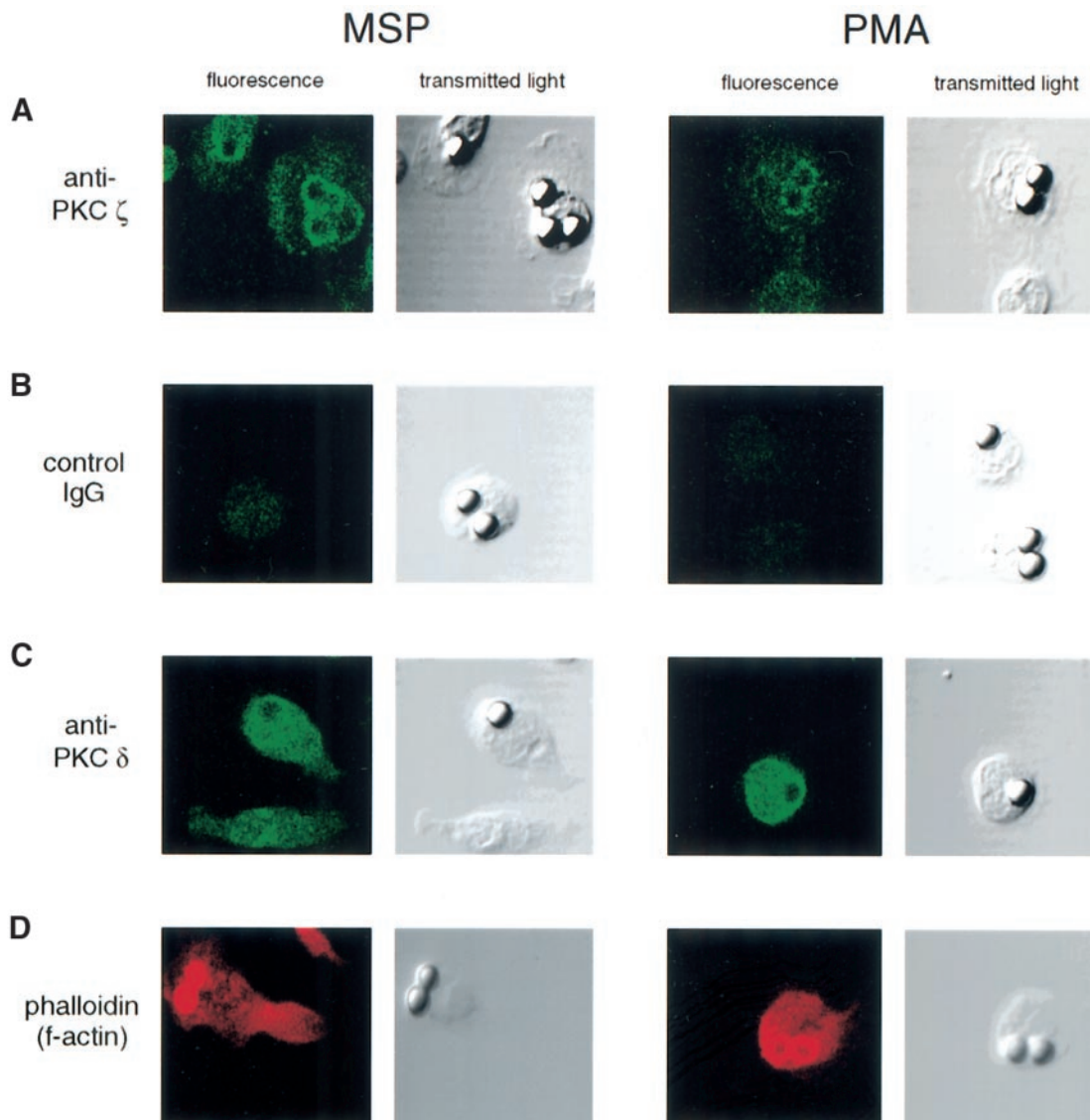


Fig. 8. PKC ζ and F-actin localize to MSP-induced phagosomes containing complement-coated latex beads. Peritoneal macrophages stimulated with 100 ng/ml MSP or 10 ng/ml PMA in the presence of complement-coated 4.5 μ latex beads were labeled with anti-PKC ζ (A), control IgG (B), anti-PKC δ (C), or phalloidin (D). Antibodies were detected by labeling with FITC-conjugated secondary antibody and were analyzed using an Olympus confocal laser-scanning microscope.

DISCUSSION

Integrin activation by RTKs has been demonstrated in many systems. Increasing evidence suggests a key role for the RON/MET family of RTKs in the activation of integrins. Hepatocyte growth factor (HGF), the ligand for the MET receptor, can induce the activation of the β 2 integrin lymphocyte function-associated antigen-1 in neutrophils, and this activation is dependent on PI-3K and tyrosine kinase activity [29]. In addition, MSP has been shown to increase β 1 integrin-dependent adhesion to ECM. Furthermore, RON and the β 1 integrin were shown to coimmunoprecipitate, suggesting a ligand-independent interaction between RON and the β 1 integrin [24]. Conversely, ECM-induced aggregation of β 1 integrins has been shown to activate the RON receptor, as evidenced by phosphorylation of RON [30]. Here, we demonstrate that the ability of MSP to induce ICAM-1 binding and CR3-mediated phagocytosis requires the RON RTK as well as the α M β 2 integrin,

as evidenced by the inability of RON $^{-/-}$ and α M null macrophages to undergo ICAM-1 binding or phagocytosis in response to MSP.

In this study, we set out to determine the signals downstream of the RON receptor required for MSP-induced CR3-mediated phagocytosis. The signals required for phagocytosis mediated by Fc receptors have been studied extensively [31–33]. However, the signaling requirements necessary for CR3-mediated phagocytosis are less clearly delineated. Conflicting data exist regarding the requirement for tyrosine kinase activity in CR3-mediated phagocytosis [27, 34]. These discrepancies may be a result of the variations in the cell type used, particle ingested, and/or mode of activation. This may also be explained by the promiscuous nature of CR3 for ligands such as ICAM-1, LPS, factor X, zymosan, as well as C3bi [4, 35] and its ability to interact with other receptors such as CD14, urokinase plasminogen activator receptor, and Fc γ IIIb [36]. Furthermore, the use of opsonized particles such as mycobacterium or yeast may

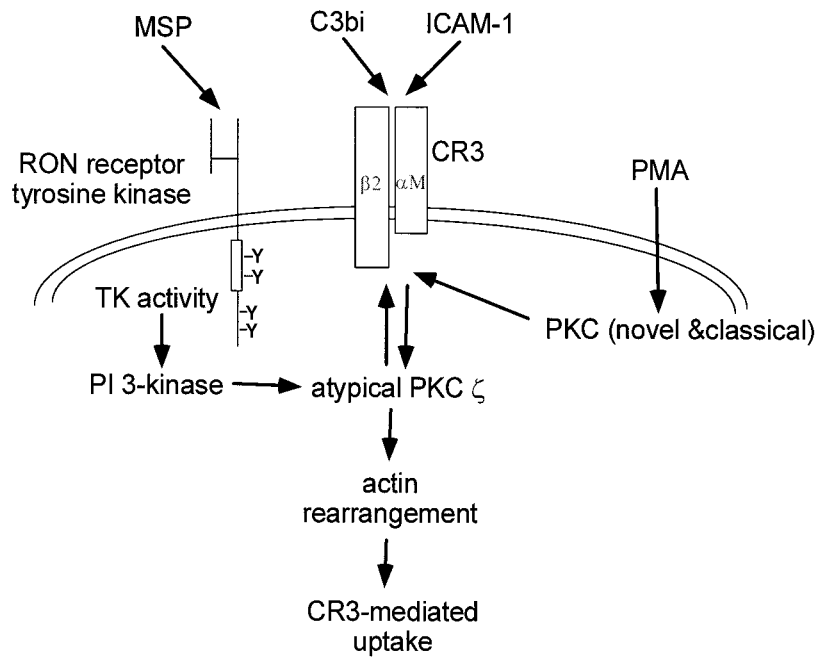


Fig. 9. The signaling requirements for MSP-induced phagocytosis and MSP-induced ICAM-1 binding in relationship to the RON receptor and CR3.

result in different signaling requirements as a result of interaction with the mannose receptor or β -glucan activation of CR3, respectively [37]. Our data suggest that in primary peritoneal macrophages, MSP-induced but not PMA-induced, complement-mediated phagocytosis and ICAM-1 binding require tyrosine kinase activity.

PI-3K is activated following receptor dimerization and autophosphorylation of RON in response to MSP, and PI-3K activation has been shown to be required for many of the biological activities induced by MSP, including epithelial cell adhesion [24], shape change and migration [38], inhibition of iNOS expression in activated macrophages [13], and transformation of Rat1 cells by v-SEA, the chicken homologue of RON [39]. Here, we demonstrate that PI-3K activation is required for MSP-induced, CR3-mediated phagocytosis and MSP-induced ICAM-1 binding. PI-3K has previously been implicated in integrin activation mediated by RTKs as well as in response to several other stimuli in leukocytes including interleukin-8, fMLP, platelet-activating factor, and TNF- α , presumably through its ability to mediate PKC-dependent activation of Rho, a downstream target of DAG-dependent and -independent PKC isoforms. Furthermore, serine phosphorylation of L-plastin and cytohesin-1, cytoplasmic regulators of β 2 integrins, is inhibited in the presence of wortmannin and LY294002 in response to a number of stimuli [3].

PI-3K-dependent activation of atypical PKCs has been demonstrated for multiple RTKs, including the insulin receptor [40] as well as epidermal growth factor receptor and platelet-derived growth factor receptor [41]. Phosphorylation of the PKC ζ activation loop site by PDK-1 in a PI-3K-dependent manner results in the activation of PKC ζ [26]. PKC ζ has been shown to play an important role in the integrin-dependent adhesion and chemotaxis of polymorphonuclear neutrophil [42] and is activated in a PI-3K-dependent manner in macrophages infected with *Salmonella* [43].

Our data also demonstrate, for the first time, a pivotal role for PKC ζ activation in complement-mediated phagocytosis in-

duced by MSP and PMA. Although PKC ζ is necessary for the upstream activation of CR3 by MSP but not PMA, our data suggest that PKC ζ is also necessary for PMA-induced phagocytosis. Furthermore, PKC ζ localizes MSP- and PMA-induced, complement-containing phagosomes, suggesting a role for PKC ζ in the downstream cytoskeletal changes during CR3-mediated phagocytosis. PKC ζ has previously been shown to induce reorganization of the actin cytoskeleton in a PI-3K-dependent manner [44] and to associate with the actin cytoskeleton [45]. PKC ζ also associates with microtubules [46] and binds to tubulin [47]. Additionally, wounding assays in astrocytes show that the interaction of integrins with the ECM leads to activation and localization of PKC ζ to a newly formed cell front [48]. PKC ζ has recently been shown to be involved in the phagocytosis of *Helicobacter pylori* [49], suggesting the possibility of a broader role for PKC ζ in the phagocytic process.

Here, we have demonstrated a role for tyrosine kinase activity, PI-3K, and PKC ζ in integrin activation in response to MSP. However, other signaling pathways have recently been identified that may also play a role in the ability of MSP/RON to activate CR3. For example, signal transducer and activator of transcription (Stat)3 has been shown to be involved in integrin activation by inducing CD18-dependent, homotypic aggregation in myeloid progenitor cells [50]. Additionally, HGF has been shown to activate Stat3 [51] through the RON-related MET receptor. We have recently demonstrated that MSP induces Stat3 tyrosine and serine phosphorylation in peritoneal macrophages (Morrison et al., submitted). Furthermore, the GTPase Rap1 has been shown to be involved in CR3 activation [52], and the GTPase Rap1 can be activated by HGF [53]. It is interesting that MET has also been shown to mediate InlB-dependent internalization of *Listeria monocytogenes* in a PI-3K, Gab1, and Cbl manner [54]. The activation of CR3-mediated phagocytosis and ICAM-1 binding by MSP/RON provides us with a system in which to further delineate the signals that regulate integrin activation by RTKs.

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REFERENCES

1. Kwiatkowska, K., Sobota, S. (1999) Signaling pathways in phagocytosis. *Bioessays* **21**, 422–431.
2. Caron, E., Hall, A. (1998) Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science* **282**, 1717–1721.
3. Williams, M. A., Solomkin, J. S. (1999) Integrin-mediated signaling in human neutrophil functioning. *J. Leukoc. Biol.* **65**, 725–736.
4. Ross, G. D., Vetvicka, V. (1993) CR3 (CD11b, CD18): a phagocyte and NK cell membrane receptor with multiple ligand specificities and functions. *Clin. Exp. Immunol.* **92**, 181–184.
5. Toker, A. (1998) Signaling through protein kinase C. *Front. Biosci.* **3**, D1134–D1147.
6. Skeel, A., Leonard, E. J. (1994) Action and target cell specificity of human macrophage-stimulating protein (MSP). *J. Immunol.* **152**, 4618–4623.
7. Wang, M-H., Yoshimura, T., Skeel, A., Leonard, E. J. (1994) Proteolytic conversion of single chain precursor macrophage-stimulating protein to a biologically active heterodimer by contact enzymes of the coagulation cascade. *J. Biol. Chem.* **269**, 3436–3440.
8. Wang, M. H., Skeel, A., Leonard, E. J. (1996) Proteolytic cleavage and activation of pro-macrophage-stimulating protein by resident peritoneal macrophage membrane proteases. *J. Clin. Invest.* **97**, 720–727.
9. Nanney, L. B., Skeel, A., Luan, J., Polis, S., Richmond, A., Wang, M. H., Leonard, E. J. (1998) Proteolytic cleavage and activation of pro-macrophage-stimulating protein and upregulation of its receptor in tissue injury. *J. Invest. Dermatol.* **111**, 573–581.
10. Skeel, A., Yoshimura, T., Showalter, S. D., Tanka, S., Appella, E., Leonard, E. J. (1991) Macrophage stimulating protein: purification, partial amino acid sequence, and cellular activity. *J. Exp. Med.* **173**, 1227–1234.
11. Wang, M-H., Cox, G. W., Yoshimura, T., Sheffer, L. A., Skeel, A., Leonard, E. J. (1994) Macrophage-stimulating protein inhibits induction of nitric oxide production by endotoxin- or cytokine-stimulated mouse macrophages. *J. Biol. Chem.* **269**, 14027–14031.
12. Liu, Q-P., Fruit, K., Ward, J., Correll, P. H. (1999) Negative regulation of macrophage activation in response to IFN- γ and lipopolysaccharide by the STK/RON receptor tyrosine kinase. *J. Immunol.* **163**, 6606–6613.
13. Chen, Y. Q., Fisher, J. H., Wang, M-H. (1998) Activation of the RON receptor tyrosine kinase inhibits inducible nitric oxide synthase (iNOS) expression by murine peritoneal exudate macrophages: phosphatidylinositol-3 kinase is required for RON-mediated inhibition of iNOS expression. *J. Immunol.* **161**, 4950–4959.
14. Ronsin, C., Muscatelli, F., Mattei, M-G., Breathnach, R. (1993) A novel putative receptor protein tyrosine kinase of the met family. *Oncogene* **8**, 1195–1202.
15. Iwama, A., Okano, K., Sudo, T., Matsuda, Y., Suda, T. (1994) Molecular cloning of a novel receptor tyrosine kinase gene, STK, derived from enriched hematopoietic stem cells. *Blood* **83**, 3160–3169.
16. Huff, J. L., Jelinek, M. A., Borgman, C. A., Lansing, T. J., Parsons, J. T. (1993) The protooncogene *c-sea* encodes a transmembrane protein-tyrosine kinase related to the Met/hepatocyte growth factor/scatter factor receptor. *Proc. Natl. Acad. Sci. USA* **90**, 6140–6144.
17. Kurihara, N., Iwama, A., Tatsumi, J., Ikeda, K., Suda, T. (1996) Macrophage-stimulating protein activates STK receptor tyrosine kinase on osteoclasts and facilitates bone resorption by osteoclast-like cells. *Blood* **87**, 3704–3710.
18. Iwama, A., Wang, M-H., Yamaguchi, N., Ohno, N., Okano, K., Sudo, T., Takeya, M., Gervais, F., Morissette, C., Leonard, E. J., et al. (1995) Terminal differentiation of murine resident peritoneal macrophages is characterized by the expression of the STK protein tyrosine kinase, a receptor for macrophage-stimulating protein. *Blood* **86**, 3394–3403.
19. Muraoka, R. S., Sun, W. Y., Colbert, M. C., Waltz, S. E., Witte, D. P., Degen, J. L., Friezner Degen, S. J. (1999) The RON/STK receptor tyrosine kinase is essential for peri-implantation development in the mouse. *J. Clin. Invest.* **103**, 1277–1285.
20. Waltz, S. E., Eaton, L., Toney-Early, K., Hess, K. A., Peace, B. E., Ihendorf, J. R., Wang, M. H., Kaestner, K. H., Degen, S. J. (2001) Ron-mediated cytoplasmic signaling is dispensable for viability but is required to limit inflammatory responses. *J. Clin. Invest.* **108**, 567–576.
21. Correll, P. H., Iwama, A., Tondat, S., Mayrhofer, G., Suda, T., Bernstein, A. (1997) Deregulated inflammatory response in mice lacking the STK/RON receptor tyrosine kinase. *Genes Funct.* **1**, 69–83.
22. Persons, D. A., Paulson, R. F., Loyd, M. R., Herley, M. T., Bodner, S. M., Bernstein, A., Correll, P. H., Ney, P. A. (1999) *Fr2* encodes a truncated form of the Stk receptor tyrosine kinase. *Nat. Genet.* **23**, 159–165.
23. Danilkovitch, A., Leonard, E. J. (1999) Kinases involved in MSP/RON signaling. *J. Leukoc. Biol.* **65**, 345–348.
24. Danilkovitch, A., Skeel, A., Leonard, E. J. (1999) Macrophage stimulating protein-induced epithelial cell adhesion is mediated by a PI3-K-dependent, but FAK-independent mechanism. *Exp. Cell Res.* **248**, 575–582.
25. Roubey, R. A., Ross, G. D., Merrill, J. T., Walton, F., Reed, W., Winchester, R. J., Buyon, J. P. (1991) Staurosporine inhibits neutrophil phagocytosis but not iC3b binding mediated by CR3 (CD11b/CD18). *J. Immunol.* **146**, 3557–3562.
26. Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., Parker, P. J. (1998) Protein kinase C isoforms controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* **281**, 2042–2045.
27. Allen, L-A., Aderem, A. (1996) Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. *J. Exp. Med.* **184**, 627–637.
28. Mera, A., Suga, M., Ando, M., Suda, T., Yamaguchi, N. (1999) Induction of cell shape changes through activation of the interleukin-3 common β chain receptor by the RON receptor-type tyrosine kinase. *J. Biol. Chem.* **274**, 15766–15774.
29. Mine, S., Tanaka, Y., Suematu, M., Aso, M., Fujisaki, T., Yamada, S., Eto, S. (1998) Hepatocyte growth factor is potent trigger of neutrophil adhesion through rapid activation of lymphocyte function-associated antigen-1. *Lab. Invest.* **78**, 1395–1404.
30. Danilkovitch-Miagkova, A., Angeloni, D., Skeel, A., Donley, S., Lerman, M., Leonard, E. J. (2000) Integrin-mediated RON growth factor receptor phosphorylation requires tyrosine kinase activity of both the receptor and *c-Src*. *J. Biol. Chem.* **275**, 14783–14786.
31. Indik, Z. K., Park, J-G., Hunter, S., Schreiber, A. D. (1995) The molecular dissection of Fc γ receptor mediated phagocytosis. *Blood* **86**, 4389–4399.
32. Daeron, M. (1997) Fc receptor biology. *Annu. Rev. Immunol.* **15**, 203–234.
33. Strzelecka, A., Kwiatkowska, K., Sobota, A. (1997) Tyrosine phosphorylation and Fc γ receptor-mediated phagocytosis. *FEBS Lett.* **400**, 11–14.
34. Kusner, D. J., Hall, C. F., Schlesinger, L. S. (1996) Activation of phospholipase D is tightly coupled to the phagocytosis of *Mycobacterium tuberculosis* or opsonized zymosan by human macrophages. *J. Exp. Med.* **184**, 585–595.
35. Arnaout, M. A. (1990) Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood* **75**, 1037–1050.
36. Todd III, R. F., Petty, H. R. (1997) $\beta 2$ (CD11/CD18) integrins can serve as signaling partners for other leukocyte receptors. *J. Lab. Clin. Med.* **129**, 492–498.
37. Linehan, S. A., Martinez-Pomares, L., Gordon, S. (2000) Macrophage lectins in host defense. *Microbes Infect.* **2**, 279–288.
38. Wang, M-H., Montero-Julian, F. A., Dauny, I., Leonard, E. J. (1996) Requirement of phosphatidylinositol-3 kinase for epithelial cell migration activated by human macrophage stimulating protein. *Oncogene* **13**, 2167–2175.
39. Park, C. Y., Hayman, M. J. (1999) The tyrosines in the bidentate motif of the env-sea oncoprotein are essential for cell transformation and are binding sites for Grb2 and the tyrosine phosphatase SHP-2. *J. Biol. Chem.* **274**, 7583–7590.
40. Mosthaf, L., Kellerer, M., Muhlhofer, A., Mushack, J., Seffer, E., Haring, H. U. (1996) Insulin leads to a parallel translocation of PI-3-kinase and protein kinase C ζ . *Exp. Clin. Endocrinol. Diabetes* **104**, 19–24.
41. Akimoto, K., Takahashi, R., Moriya, S., Nishioka, N., Takayanagi, J., Kimura, K., Fukui, Y., Osada, S., Mizuno, K., Hirai, S., Kazlauskas, A., Ohno, S. (1996) EGF or PDGF receptors activate atypical PKC λ through phosphatidylinositol 3-kinase. *EMBO J.* **15**, 788–793.
42. Laudanna, C., Mochly-Rosen, D., Liron, T., Costantin, G., Butcher, E. C. (1998) Evidence of ζ protein kinase C involvement in polymorphonuclear neutrophil integrin-dependent adhesion and chemotaxis. *J. Biol. Chem.* **273**, 30306–30315.
43. Procyk, K. J., Rippo, M. R., Testi, R., Hoffman, F., Parker, P. J., Baccarini, M. (1999) Distinct mechanisms target stress and extracellular signal-activated kinase-1 and Jun N-terminal kinase during infection of macrophages with *Salmonella*. *J. Immunol.* **163**, 4924–4930.
44. Uberall, F., Hellbert, K., Kampfer, S., Maly, K., Villunger, A., Spitaler, M., Mwanjewe, J., Baier-Bitterlich, G., Baier, G., Grunicke, H. H. (1999)

- Evidence that atypical protein kinase C- λ and atypical protein kinase C- ζ participate in Ras-mediated reorganization of the F-actin cytoskeleton. *J. Cell Biol.* **144**, 413–425.
45. Gomez, J., de Aragon, A. M., Bonay, P., Pitton, C., Garcia, A., Silva, A., Fresno, M., Alvarez, F., Rebollo, A. (1995) Physical association and functional relationship between protein kinase C ζ and the actin cytoskeleton. *Eur. J. Immunol.* **25**, 2673–2678.
 46. Lehrich, R. W., Forrest Jr., J. N. (1994) Protein kinase C ζ is associated with the mitotic apparatus in primary cell cultures of the shark rectal gland. *J. Biol. Chem.* **269**, 32446–32450.
 47. Garcia-Rocha, M., Avila, J., Lozano, J. (1997) The ζ isozyme of protein kinase C binds to tubulin through the pseudosubstrate domain. *Exp. Cell Res.* **230**, 1–8.
 48. Etienne-Manneville, S., Hall, A. (2001) Integrin-mediated activation of cdc42 controls cell polarity in migrating astrocytes through PKC ζ . *Cell* **106**, 489–498.
 49. Allen, L. H., Allgood, J. A. (2002) Atypical protein kinase C-zeta is essential for delayed phagocytosis of *Helicobacter pylori*. *Curr. Biol.* **12**, 1762–1766.
 50. Wooten, D. K., Xie, X., Bartos, D., Busche, R. A., Longmore, G. D., Watowich, S. S. (2000) Cytokine signaling through Stat3 activates integrins, promotes adhesion, and induces growth arrest in the myeloid cell line 32D. *J. Biol. Chem.* **275**, 26566–26575.
 51. Schaper, F., Siewert, E., Gomez-Lechon, M. J., Gatsios, P., Sachs, M., Birchmeier, W., Heinrich, P. C., Castell, J. (1997) Hepatocyte growth factor/scatter factor (HGF/SF) signals via the STAT3/APRF transcription factor in human hepatoma cells and hepatocytes. *FEBS Lett.* **405**, 99–103.
 52. Caron, E., Self, A. J., Hall, A. (2000) The GTPase Rap1 controls functional activation of macrophage integrin $\alpha M\beta 2$ by LPS and other inflammatory mediators. *Curr. Biol.* **10**, 974–978.
 53. Sakkab, D., Lewitzky, M., Posern, G., Schaeper, U., Sachs, M., Birchmeier, W., Feller, S. M. (2000) Signaling of hepatocyte growth factor/scatter factor (HGF) to the small GTPase Rap1 via the large docking protein Gab1 and the adapter protein CRKL. *J. Biol. Chem.* **275**, 10772–10778.
 54. Shen, Y., Naujokas, M., Park, M., Ireton, K. (2000) INIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. *Cell* **103**, 501–510.