Inhibition of TLR4-Induced IκB Kinase Activity by the RON Receptor Tyrosine Kinase and Its Ligand, Macrophage-Stimulating Protein

Manujendra Ray[*,†,1], **Shan Yu**[*,‡,1], **Daniel R. Sharda**[*,§], **Caleph B. Wilson**[*,§], **QingPing Liu**[*], **Naveen Kaushal**[*], **K. Sandeep Prabhu**[*,†,§], and **Pamela A. Hankey**[*,†,‡,§]

[*]Department of Veterinary and Biomedical Science, The Pennsylvania State University, University Park, PA 16802

[†]Graduate Program in Immunology and Infectious Disease, The Pennsylvania State University, University Park, PA 16802

[‡]Graduate Program in Physiology, The Pennsylvania State University, University Park, PA 16802

[§]Graduate Program in Pathobiology, The Pennsylvania State University, University Park, PA 16802

Abstract

The RON receptor tyrosine kinase regulates the balance between classical (M1) and alternative (M2) macrophage activation. In primary macrophages, the ligand for Ron, macrophage-stimulating protein (MSP), inhibits the expression of inducible NO synthase, a marker of classically activated macrophages, whereas promoting the expression of arginase I, a marker of alternative activation. Ron$^{-/-}$ mice express increased levels of IL-12, a product of classically activated macrophages, after endotoxin administration, resulting in increased serum IFN-γ levels and enhanced susceptibility to septic shock. In this study, we demonstrate that MSP inhibits LPS-induced IL-12p40 expression, and this inhibition is dependent on the docking site tyrosines in Ron. To further define this inhibition, we examined the effect of Ron on signaling pathways downstream of Ron. We found that MSP does not inhibit the MyD88-independent activation of IFN regulatory factor 3 and production of IFN-β in response to LPS, nor does it inhibit MyD88-dependent TGF-β–activated kinase phosphorylation or MAPK activation in primary macrophages. However, the induction of IκB kinase activity, IκB degradation, and DNA binding of NF-κB after LPS stimulation is delayed in the presence of MSP. In addition, Ron inhibits serine phosphorylation of p65 and NF-κB transcriptional activity induced by LPS stimulation of TLR4. Finally, MSP inhibits the NF-κB–dependent upregulation of the nuclear IκB family member, IκBζ, a positive regulator of secondary response genes including IL-12p40. LPS also induces expression of Ron and an N-terminally truncated form of Ron, Sf-Ron, in primary macrophages, suggesting that the upregulation of Ron by LPS could provide classical feedback regulation of TLR signaling.

Address correspondence and reprint requests to Dr. Pamela A. Hankey, Professor of Immunology, Department of Veterinary and Biomedical Science, The Pennsylvania State University, 115 Henning Building, University Park, PA 16802-3500. phc7@psu.edu.
[1]M.R. and S.Y. contributed equally to this work.

Introduction

Macrophages are a heterogeneous population of cells that exhibit a wide range of functions depending on their physical location, as well as external cues received from the tissue microenvironment (1, 2). Classical, or M1, macrophages are the most commonly described subset of macrophages. These macrophages are activated by proinflammatory cytokines and pathogen-associated molecular patterns that result in stimulation of pattern recognition receptors including the family of TLRs. Classically activated macrophages, in turn, produce proinflammatory cytokines and an array of cytotoxic molecules that aid in the clearance of invading pathogens and stimulate the acquired immune response. Alternative, or M2, macrophages, in contrast, develop in response to Th2 cytokines and produce anti-inflammatory cytokines, express an array of phagocytic receptors, and release products that promote tissue regeneration and healing. The characteristic hallmarks of these diverse macrophage populations are typified by the expression of inducible NO synthase (iNOS) and arginase I by M1 and M2 macrophages, respectively. These enzymes share a common substrate, l-arginine, but whereas iNOS promotes the production of NO, which is cytotoxic, arginase promotes the production of polyamines and proline, which promote the healing process.

Although classically activated macrophages promote immunity to infection, products from these cells can also be damaging to host tissues and are implicated in the progression of a number of chronic inflammatory diseases. Thus, the study of signals that tip the balance of macrophage activation away from the M1 phenotype and toward the M2 phenotype could have wide-ranging therapeutic implications. A critical role for receptor tyrosine kinases in the regulation of macrophage activation is emerging (3, 4). The RON receptor tyrosine kinase is expressed by tissue-resident macrophages and inhibits classical macrophage activation whereas promoting hallmarks of alternative macrophage activation. Macrophage-stimulating protein (MSP), the ligand for the RON receptor, regulates arginine metabolism in macrophages by inhibiting the expression of iNOS and promoting the expression of arginase in primary resident peritoneal macrophages (5–7). Furthermore, mice with a targeted deletion in the gene encoding Ron exhibit enhanced inflammation and tissue damage in models of septic shock, delayed type hypersensitivity, experimental autoimmune encephalitis, and acute lung injury (8–11). Thus, understanding the mechanism by which the RON receptor promotes these responses is of potential clinical significance.

We have shown previously that the RON receptor is a critical regulator of IL-12 production by macrophages both in vitro and in vivo (12, 13). IL-12, a product of classically activated macrophages, plays a central role in promoting both the innate and acquired immune response to infection (14). In the early stages of infection, IL-12 produced by macrophages induces the expression of IFN-γ by NK cells, $\gamma\delta$ T cells, and innate CD8+ T cells. At later stages, IL-12 promotes the differentiation of Th1 cells, which produce increased levels of IFN-γ and drive the acquired immune response to infection. IFN-γ produced by both innate and acquired immune cells feeds back to prime macrophages for further activation of the M1 phenotype. Our previous studies demonstrate that enhanced production of IL-12 by Ron–/– splenocytes after injection with LPS, a ligand for TLR4, results in increased IFN-γ production by NK cells and enhanced susceptibility of Ron–/– mice to endotoxic shock.

Furthermore, MSP stimulation of the RON receptor on primary macrophages inhibits the response of these cells to IFN-γ through inhibition of STAT1 tyrosine phosphorylation induced by IFN-γ. MSP stimulation of primary macrophages induces the expression of suppressor of cytokine signaling (Socs) 1 and Socs3 delineating a mechanism by which the inhibition of IFN-γ–dependent responses in these cells could occur (13).

Several studies have demonstrated that the RON receptor inhibits LPS and cytokine-induced NF-κB activation in a variety of macrophage populations (6, 15, 16); however, the molecular targets of Ron downstream of TLR activation in primary macrophages have not been systematically addressed. In this report, we show that stimulation of primary peritoneal macrophages with MSP, the ligand for Ron, does not inhibit receptor proximal signaling events initiated by LPS stimulation of TLR4, but rather specifically inhibits the activation of IκB kinase (IKK) and its downstream targets by LPS. Furthermore, we demonstrate the induction of Ron expression by LPS, identifying this as a potential negative feedback response for regulating TLR4 signaling. These studies will contribute to the identification of potential regulatory mechanisms that can be targeted in the treatment of a wide range of inflammatory diseases.

Materials and Methods

Animals

The generation of mice with a targeted mutation in Ron was described previously (8). Primary peritoneal macrophages or bone marrow-derived macrophages were isolated from wild-type and Ron−/− mice on the BALB/c or C57BL/6 backgrounds. The Pennsylvania State University Institutional Animal Care and Use Committee approved all animal experiments.

Reagents

The following reagents were obtained from the indicated sources: FBS and PBS (Invitrogen, Grand Island, NY); recombinant human MSP (R&D Systems, Minneapolis, MN); LPS (055:B5; Sigma, St. Louis, MO); DMEM, l-glutamine, and nonessential amino acids and sodium pyruvate (cellgro; Mediatech, Manassas, VA). Abs for Western blot against IκBα, phosphoS396IFN regulatory factor 3 (IRF3), total IRF3, phosphoThr184/187TGF-β–activated kinase (Tak1), total Tak1, IkBζ, phosphoT202Y204p44/42, total p44/42, phosphoT183Y185stress-activated protein kinase/JNK, total stress-activated protein kinase/JNK, phosphoT180Y182p38, total p38, phosphoY307Gab1, total Gab1, phosphoY452Gab2, total Gab2, phosphoS536p65, total p65, and hemagglutinin (HA)-tag were purchased from Cell Signaling (Beverly, MA). Anti-Bcl3 (C-14) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Abs for flow cytometry, F4/80-R-phycoerythrin, CD11b-allophycocyanin-750, and Ron were purchased from AbD Serotec (Raleigh, NC), eBioscience (San Diego, CA), and BD Biosciences (San Jose, CA), respectively.

Cells

Murine resident peritoneal macrophages were harvested and cultured as described previously (17). Thioglycollate-elicited macrophages were prepared by injecting 2-mo-old

BALB/c mice with 2 ml 3% aged thioglycollate media (Difco, Sparks, MD). On day 4, cells were harvested by lavage, and macrophages were isolated and cultured as described for resident macrophages. 293 cells stably transfected with TLR4 and MD-2 (a kind gift from Dr. Golenbock, University of Massachusetts, Worcester, MA) were maintained in complete cell culture medium (DMEM with l-glutamine, nonessential amino acids, sodium pyruvate, ciprofloxacin, and 10% FBS) supplemented with 0.5 mg/ml G418. Bone marrow-derived macrophages were differentiated from progenitors in the bone marrow by culturing bone marrow cells in macrophage differentiation media (30% L929 conditioned media, 20% FBS, 50% DMEM) for 5 d.

Luciferase assays

293 cells stably expressing TLR4 and MD-2 were plated overnight in 24-well plates and then transfected using the Mirus transfection reagent (Mirus Bio, Madison, WI), according to the manufacturer's recommendations. The cells were transfected with 5 ng ELAM or AP-1 luciferase and the indicated concentrations of PCDNA3.1 or PCDNA3.1-Ron-HA. Twenty-four hours after transfection, the cells were stimulated for 4 h with 100 ng/ml LPS. Cell lysates were harvested and the luciferase assay was performed using a Turner Designs TD-20/20 luminometer (Sunnyvale, CA). Luciferase assay reagents were purchased from Promega (Madison, WI).

Retroviral transduction

Bone marrow-derived macrophages were infected twice (once for 6 h, followed by another round of infection overnight) with media containing 75% retroviral supernatants, 25% fresh complete media, and 5 μg/ml polybrene. Viral supernatants were generated by transiently transfecting 293 cells using the Mirus reagent with pEco, pVsvg, and murine stem cell virus-based vectors containing the indicated cDNAs. Twenty-four hours after transfection, the media was changed, and after another 24 h, the culture supernatants containing the packaged virus were collected and filtered.

Cytokine analysis

Cells were cultured with or without 100 ng/ml MSP overnight followed by stimulation for 24 h with 100 ng/ml LPS. Cell supernatants were collected and assayed for IL-12p40 by ELISA using the manufacturer's protocol (BD Biosciences, San Jose, CA).

Western blot analysis

Cell lysates were collected using the following lysis buffer (150 mM NaCl, 10 mM Tri-HCl [pH 7.5], 0.5% Triton X-100), supplemented with protease and phosphatase inhibitors (10 μg/ml leupeptin, 2 μg/ml pepstatin A, 10 μg/ml aprotinin, 1 mM sodium orthovanadate, 1 M sodium fluoride, and 1 mM PMSF). SDS-PAGE was performed using 10% bis-acrylamide gels. The protein was transferred onto Immobilon P polyvinylidene difluoride membranes (Millipore, Bedford, MA), and the blots were blocked with 5% nonfat dry milk or 5% BSA (Rockland, Gilbertsville, PA) in TBS containing 0.1% Tween 20 for 1 h at room temperature. The blots were then incubated overnight with primary Ab at 4°C. Anti-rabbit or anti-mouse secondary Abs conjugated with HRP were used at concentrations according to

the manufacturer's instructions for 1 h at room temperature. ECL Plus (Amersham, Piscataway, NJ) was used to develop the blots.

In vitro kinase assay

Cell lysates from thioglycollate-elicited macrophages were prepared using the M-PER buffer (Pierce, Rockford, IL) with protease and phosphatase inhibitors as described earlier. The cell lysates (100 μg protein) were incubated with GST-IκBα, ATP, and [γ-32P]ATP (1 μCi; Amersham Biosciences) for 1.5 h in the kinase wash buffer (50 mM Tris-Cl, pH 8.0, containing 100 μM NaCl, 10 mM MgCl2, 1 mM DTT, 10 mM β-glycerophosphate, 10 mM NaF, and 1 mM sodium orthovanadate) on a shaker at room temperature. Glutathione Sepharose beads were added to the mixture and incubated at room temperature for 1 h with gentle shaking followed by centrifugation. The pellet was washed two times with sterile PBS and phospholabeled GST-IκB was separated by SDS-PAGE, followed by autoradiography.

Electrophoretic mobility shift assay

Nuclear proteins were isolated from thioglycollate-elicited macrophages as described previously (18). The DNA sequence of the sense strand specific for NF-κB was 5′-AGTTGAGGGGACTTTCCCAGGC-3′, and the complementary strand was annealed. The double-stranded oligonucleotides were labeled with [γ-32P]ATP (3000 Ci/mmol and 10 mCi/ml) by the T4 polynucleotide kinase (NEB, Beverly, MA). Ten micrograms of nuclear proteins was incubated in a binding buffer specific for NF-κB as described previously (19) in the presence of 2 μg poly(dI-dC) for 20 min at room temperature. The nuclear extract was then incubated for 20 min with 10,000 cpm 32P-labeled NF-κB probes, and the samples were analyzed as described previously (18).

NF-κB binding assay

Thioglycollate-elicited macrophages were cultured with or without 100 ng/ml MSP overnight followed by stimulation with 100 ng/ml LPS for the indicated times. Nuclear proteins were collected and assayed for NF-κB binding activity by ELISA according to the manufacturer's protocol (Active Motif, Carlsbad, CA).

Flow cytometry

Peritoneal lavage cells were plated overnight with or without MSP stimulation. Twelve hours after adherence to the plate, cells were collected. One × 106 cells were incubated per tube in 100 μl FACS buffer (ice-cold PBS + 2% FBS). Nonspecific binding was blocked by the addition of 1 μl Fc-block for 10 min on ice. F4/80-R-phycoerythrin, CD11b-allophycocyanin-750, and Ron Abs were added to the tubes for 30 min on ice. Cells were washed two times with FACS buffer and resuspended in 100 μl FACS buffer. Cells were incubated with anti-goat Alexa 647 as a secondary Ab to Ron for 30 min on ice. Cells were washed two more times, resuspended in 500 μl FACS buffer, and analyzed on a Beckman Coulter FC500 (Fullerton, CA). Ron expression was measured in macrophages positive for F4/80 and CD11b.

RNA extraction and real-time PCR

RNA was extracted from cultured cells using the RNeasy mini column kit (Qiagen, Santa Clara, CA). RNA was treated with DNase 1 (Invitrogen) according to the manufacturer's recommendations. Real-time PCR was carried out on Applied Biosystems 7300 (San Francisco, CA). Reverse transcription for real-time PCR experiments was carried out using the Applied Biosystems High Capacity RT Kit according to the manufacturer's recommendations. Gene-specific primers and probes were designed by the Nuclei Acid Facility at the Pennsylvania State University using Primer Express V.1.0 (Applied Biosystems). Primer and probe sequences for Ron were described previously (13). Primer and probe sequences for Sf-Ron are as follows: forward 5′-ACTGTGAACATGACCGTGGGT-3′, reverse 5′-TGCCAAGTTGCAGGGAAGG-3′, and probe 5′6-FAM d(AGGTCTGCCAACATGAGCTCCGG)BHQ-1-3′. GAPDH was used as the internal standard (GAPDH primer and probe set; Applied Biosystems). IL-12p40 primer and probe sets were purchased from Applied Biosystems.

Statistical analysis

Statistical analyses were performed using the Student t test.

Results

MSP stimulation of the RON receptor inhibits IL-12p40 production by primary macrophages in response to LPS

We have shown previously that MSP stimulation of primary peritoneal macrophages inhibits the induction of IL-12p40 expression in response to IFN-γ and LPS. This inhibition is associated with reduced IFN-γ–dependent STAT1 phosphorylation and IFN consensus sequence binding protein upregulation, and the upregulation of Socs1 and Socs3 by MSP (12). However, the effect of the MSP/Ron signaling pathway specifically on LPS/TLR4 signaling in primary macrophages has not been investigated. In this study, we cultured primary peritoneal macrophages overnight in the presence of MSP, followed by stimulation with LPS in the presence or absence of MSP, and assessed IL-12p40 production by ELISA (Fig. 1A). Results from these studies demonstrate that MSP inhibits IL-12p40 expression in response to LPS stimulation after overnight culture in the absence of MSP. However, this inhibition was significantly enhanced when the cells were first cultured overnight in the presence of MSP. The sustained expression of Ron on primary macrophages after overnight culture was confirmed by flow cytometry (Supplemental Fig. 1). These conditions were used for all subsequent experiments.

To determine whether this inhibition is mediated by the RON receptor, we treated primary macrophages from wild-type and Ron−/− mice with MSP before stimulation with IFN-γ. Results from these studies demonstrate that the inhibition of IL-12p40 by MSP is dependent on the RON receptor (Fig. 1B). To confirm that MSP induces Ron-dependent signaling in primary macrophages, we examined the phosphorylation of Gab1 and Gab2, two adaptors that are recruited to Ron and mediate signaling downstream of Ron. We show that MSP induces robust phosphorylation of both Gab1 and Gab2 in primary macrophages, and this phosphorylation is dependent on the RON receptor (Fig. 1C). To determine whether the

inhibition of IL-12p40 by Ron occurs at the RNA level, we treated primary peritoneal macrophages with MSP overnight, followed by stimulation with LPS for various times, and assessed IL-12p40 mRNA expression by real-time PCR (Fig. 1D). Results from these studies demonstrate that IL-12p40 RNA is induced within 2 h by LPS, peaking at 4 h poststimulation, and that this induction is inhibited by MSP.

To confirm that signals generated by the RON receptor mediate the inhibition of LPS-induced IL-12p40 production, we used retroviral vectors to express Ron infected primary bone marrow-derived macrophages that do not express endogenous Ron. The infected cells were stimulated with LPS in the presence or absence of MSP, and the production of IL-12p40 was assessed by ELISA. Under these conditions, expression of Ron alone inhibited IL-12p40 production in response to LPS, and this inhibition was enhanced in the presence of MSP. Mutation of both C-terminal docking site tyrosines to phenylalanine in Ron abrogated the inhibition of LPS-induced IL-12p40 production in either the presence or absence of MSP (Fig. 2A). Interestingly, mutation of the first docking site tyrosine (Y1330F) resulted in the loss of inhibition in the absence of ligand, but retention of inhibitory activity in the presence of MSP. These results indicate that signals emanating from the docking site tyrosines in Ron are responsible for the inhibition of IL-12p40 after LPS stimulation.

The second docking site tyrosine in Ron binds to Grb2, resulting in the subsequent recruitment of Gab1 and Gab2. To assess the potential role of Grb2 in the inhibition of LPS-induced IL-12p40 production by MSP, we used primary resident macrophages from Grb2 heterozygous mice (homozygous mutant animals are embryonic lethal). Using these animals, we have shown previously that Grb2 plays an essential role in the progression of erythroleukemia induced by a truncated form of the RON receptor (20). Resident macrophages from Grb2+/− mice or their littermate controls were cultured in the presence or absence of 100 ng/ml MSP for 12 h, followed by stimulation with 100 ng/ml LPS for 24 h. IL-12p40 levels in the supernatants were assessed by ELISA. We observed a statistically significant difference in the level of IL-12p40 suppression by MSP between wild-type and Grb2+/− macrophages indicating that the recruitment of Grb2 to the second docking site tyrosine in Ron may play a role in the inhibition of LPS-induced IL-12p40 after MSP stimulation of Ron (Fig. 2B).

MSP does not inhibit receptor proximal signaling by LPS/TLR4

TLR4 signals through MyD88-dependent and -independent pathways. MyD88-independent signaling by TLR4 requires the adaptor proteins TRIF and TRIF-related adaptor molecule, and results in the phosphorylation of IRF3 and the upregulation of IFN-β. The IFN-β produced in response to TLR4 activation results in delayed activation of STAT1 phosphorylation, which promotes the expression of a secondary wave of LPS-responsive genes, including IL-12p40 (21). To determine whether MSP inhibits the MyD88-independent signaling pathway induced by TLR4, we treated primary peritoneal macrophages with MSP followed by stimulation with LPS, and the phosphorylation of IRF3 and upregulation of IFN-β were assessed. Our results indicate that MSP does not inhibit the serine phosphorylation of IRF3 in response to LPS (Fig. 3A) or the upregulation of IFN-β as assessed by RT-PCR (Fig. 3B) and ELISA (Fig. 3C).

The TLR4-dependent activation of MAPK signaling promotes the expression of many LPS-dependent genes. Alternatively, recent studies demonstrate that hyperactivation of the ERK signaling pathway by LPS can inhibit IL-12p40 expression through the activation of a repressor element in the IL-12p40 promoter (22). LPS stimulation of TLR4 induces MAPK signaling through the recruitment and activation of the adaptors MyD88 and Mal, and the upregulation of Tak1 kinase activity. We have shown previously that MSP induces robust phosphorylation of ERK in primary peritoneal macrophages (13); however, the effect of MSP on the induction of MAPK signaling in response to LPS has not been assessed. To address this question, we stimulated primary peritoneal macrophages with MSP followed by LPS stimulation. Phosphorylation of ERK, p38, and JNK was assessed at various times after LPS stimulation (Fig. 4A). Results from these studies indicate that LPS induces the phosphorylation of ERK, p38, and JNK in primary macrophages, and this induction is not inhibited by MSP. In fact, we consistently observed an increase in the phosphorylation of these MAPK family members in the presence of MSP.

MAPK signaling promotes inflammatory gene expression, at least in part, through the induction of the transcription factor AP-1. To determine whether Ron likewise induces AP-1 transcriptional activity downstream of LPS/TLR4, we used human embryonic kidney 293 (HEK293) cells stably transfected with TLR4 and MD-2. These cells were transiently transfected with CD14, with or without Ron, and an AP-1 luciferase reporter. The cells were stimulated with or without LPS and luciferase activity was assessed (Fig. 4B). As we have observed previously, Ron induces ligand-independent AP-1 reporter activity in these cells (23, 24). LPS stimulation of TLR4 also induced AP-1 reporter activity, and the combination of Ron and LPS induced levels of AP-1 activity greater than those observed with either Ron or LPS alone. These results indicate that Ron does not inhibit, but rather enhances, LPS-induced MAPK signaling and the upregulation of AP-1 transcriptional activity induced by LPS.

MSP inhibits LPS-induced activation of IKK downstream of Tak1

We have shown previously that Ron inhibits NF-κB activity in primary human monocyte-derived macrophages and RAW264.7 cells transfected with exogenous Ron (6, 15). However, the effect of endogenous Ron expressed in primary peritoneal macrophages on NF-κB signaling and the level at which this regulation occurs have not been assessed. Activation of Tak1 kinase in response to TLR4 mediates the activation of both the MAPK and NF-κB signaling pathways. Therefore, we assessed the effect of MSP on the phosphorylation of Tak1 (Fig. 5A). Consistent with the failure of MSP to inhibit MAPK signaling in response to LPS, we failed to detect a reduction in Tak1 phosphorylation by LPS in the presence of MSP. To determine whether Ron regulates IKK activity induced by LPS in primary macrophages, we used thioglycolate-elicited macrophages. We have confirmed that these cells express Ron, and that MSP inhibits LPS-induced IL-12p40 expression in these cells (data not shown). Elicited macrophages were stimulated with LPS in the presence or absence of MSP, and IKK activity was assessed by in vitro kinase assay using IκB as a substrate (Fig. 5B). We observed a significant upregulation of IKK activity within 15 min of LPS stimulation in these cells. However, this induction was significantly

reduced in the presence of MSP. IKK activity returned to basal levels by 60 min in both the presence and absence of MSP.

IKK phosphorylation of cytoplasmic IκB family members results in the proteasome-mediated degradation of IκB. We next examined IκBα protein levels in primary resident macrophages stimulated with LPS in the presence or absence of MSP (Fig. 5C). We observed a reduced rate of IκB degradation in the presence of MSP; however, by 15 min, IκB protein was undetectable in LPS-stimulated cells in either the presence or absence of MSP. To examine the effect of MSP on LPS-induced NF-κB DNA binding, we extracted nuclear lysates from elicited macrophages stimulated with LPS in the presence or absence of MSP. DNA binding of p65 from nuclear lysates was assessed by ELISA (Fig. 5D). Consistent with the IKK and IκB results, we observed a significant reduction in the DNA binding of NF-κB at 15 min after LPS stimulation in the presence of MSP. These results support our previous observations and suggest that MSP delays the activation of NF-κB signaling through the canonical pathway after stimulation of primary macrophages with LPS.

Ron inhibits serine phosphorylation of p65 and NF-κB transcriptional activity

In addition to its role in the phosphorylation of IκB, the IKK complex also promotes phosphorylation of p65 resulting in enhanced transcriptional activity. To determine whether Ron regulates NF-κB transcriptional activity, we used HEK293 cells stably expressing TLR4 and MD-2. These cells were transiently transfected with CD14 and an NF-κB luciferase reporter in the presence of increasing levels of Ron, and luciferase activity was assessed (Fig. 6A). In this system, we observed a significant reduction in NF-κB–dependent reporter activity in response to LPS in the presence of Ron in a dose-dependent manner. Similar results were observed with both murine and human Ron (data not shown).

Ron inhibits NF-κB transcriptional activity and p65 serine phosphorylation in response to LPS. A, HEK293 cells expressing TLR4 and MD-2 were cotransfected with the same empty vector or HA-tagged Ron and an NF-κB luciferase reporter. The transfected cells were stimulated with 100 ng/ml LPS and luciferase activity was assessed. Lysates were also analyzed by Western blot using an anti-HA Ab. B, HEK293 cells expressing TLR4 and MD-2 were transfected with or without Ron and stimulated with 100 ng/ml LPS. Nuclear lysates were isolated and phosphorylation of p65 at serine 536 was assessed by Western blot analysis using a phosphospecific Ab. Blots were stripped and reprobed with anti-p65 and anti-Sp1.

Phosphorylation of serine 536 on p65 by IKK enhances its transcriptional activity by promoting its association with the transcriptional activator CREB-binding protein/p300 and a member of the basal transcriptional machinery, TAFII31 (25, 26). To determine whether Ron regulates serine phosphorylation of p65 in these cells, HEK293 cells expressing TLR4 and MD-2 were transiently transfected with or without Ron and stimulated with LPS. Nuclear extracts were isolated, and phosphorylation of p65 at serine 536 was assessed by Western blot analysis using a phosphospecific Ab (Fig. 6B). In these cells, we observed a decrease in serine phosphorylation of p65 induced by LPS in the presence of MSP. These results indicate that Ron regulates the transcriptional activity of NF-κB at the level of

posttranslational modification, by limiting serine phosphorylation of p65. Consistent with these results, inhibition of p65 serine phosphorylation was also seen in primary peritoneal macrophages within 15 min of LPS stimulation (Fig. 7B).

MSP inhibits NF-κB transcriptional activity and delays serine phosphorylation of p65 in primary macrophages. A, Primary resident peritoneal macrophages were harvested by lavage and isolated by adherence to plastic. The cells were stimulated with 100 ng/ml MPS overnight followed by 100 ng/ml LPS. Cell lysates were harvested at the indicated times, and IκBζ expression was assessed by Western blot analysis. The blot was stripped and reprobed with anti-actin. B, Primary resident peritoneal macrophages were stimulated with LPS for the indicated times in the presence or absence of MSP. Serine phosphorylation of p65 was assessed by Western blot analysis.

Unlike conventional IκB family members, nuclear IκBs contain a nuclear localization signal and reside primarily in the nucleus, rather than the cytoplasm. These IκB family members exhibit both positive and negative effects on NF-κB–dependent transcriptional activation (27). IκBζ is a nuclear IκB family member that is upregulated at the transcriptional level by LPS in an NF-κB–dependent manner (28). IκBζ-deficient macrophages display impaired expression of a subset of LPS-inducible genes including IL-12p40 (29). To examine the effect of MSP on the induction of early NF-κB response genes, we assessed the expression of IκBζ in response to LPS in the presence or absence of MSP in primary peritoneal macrophages (Fig. 7A). Results from these studies demonstrate a significant reduction in LPS-induced IκBζ expression in the presence of MSP.

LPS induces Ron and Sf-Ron expression in primary peritoneal macrophages

Our in vitro and in vivo data indicate that Ron is a critical regulator of LPS/TLR4 signaling in macrophages. The gene encoding Ron contains an internal promoter that drives expression of an N-terminally truncated form of Ron that lacks the extracellular ligand binding domain but retains the transmembrane and kinase domains (Sf-Ron). The inflammatory defects observed in mice harboring an Sf-Ron–specific deletion support the concept that, like the full-length RON receptor, Sf-Ron also plays a critical role in the regulation of macrophage activation (30). To determine whether LPS regulates the expression of Ron and/or Sf-Ron in macrophages, we stimulated primary peritoneal macrophages with LPS and examined Ron and Sf-Ron expression by real-time PCR (Fig. 8). Results from these studies indicate that LPS induces expression of both Ron and Sf-Ron in primary macrophages. These results are consistent with a recent report describing two NF-κB sites in the promoter of Ron that promote its expression (31), and suggest that the upregulation of Ron by LPS could serve as a negative feedback signal to limit subsequent LPS stimulation of primary macrophages.

Discussion

Previous studies from our laboratory indicate that the MSP/Ron signaling pathway profoundly impacts the expression of IL-12p40 both in vitro and in vivo, rendering Ron−/− mice more susceptible to septic shock (12, 13). MSP stimulation of the RON receptor also inhibits the expression of iNOS and cyclooxygenase-2 in activated macrophages (5, 6, 32).

However, our previous studies also demonstrated that, whereas the production of TNF-α is inhibited in activated macrophages by MSP, IL-1β and IL-10 production by these cells is unaffected by MSP, and IL-6 production by these cells is enhanced in the presence of MSP (12). Taken together, these studies indicate that Ron differentially regulates LPS-dependent gene expression. In this study, we demonstrate that MSP stimulation of Ron does not inhibit LPS-induced TLR4 signaling at a receptor proximal level, but rather inhibits TLR signaling at the level of IKK activity. These results are consistent with a role for the RON receptor in shaping the repertoire of LPS-dependent responses in macrophages.

The data presented in this study indicate that the ability of MSP to inhibit LPS signaling in primary macrophages is enhanced significantly when the cells are cultured overnight in MSP before LPS stimulation. These results suggest that the ability of Ron to promote new protein synthesis plays a central role in the inhibition of LPS signaling by MSP. We have shown previously that MSP stimulation of the RON receptor promotes STAT3 tyrosine phosphorylation in resident macrophages (13). Our studies suggest that the activation of STAT3 by Ron occurs via the recruitment of a Grb2/Gab2 signaling complex to Ron, and the subsequent recruitment of STAT3 to the complex through a YxxQ motif present in Gab2 (33). STAT3 promotes a gene expression program, the products of which inhibit proinflammatory mediator induction. For example, Stat3 induces expression of a number of anti-inflammatory genes including the transcriptional repressor and corepressor, ETV3 and SBNO2. These repressors mediate inhibition of NF-κB–activated transcriptional reporters (34). Thus, the activation of STAT3 by MSP/Ron could induce the expression of anti-inflammatory genes that subsequently inhibit NF-κB–dependent gene expression.

We have shown previously that MSP induces the expression of Socs1 and Socs3 in primary macrophages (13). Expression of Socs3 in macrophages is induced by STAT3 (35). Socs1 inhibits IFN-induced STAT1 phosphorylation by inhibiting Jak2 kinases, via kinase inhibitory region domain, and inducing the ubiquitin-mediated degradation of this kinase (35). It has also been demonstrated that phosphorylation of the adaptor Mal promotes its interaction with Socs1 in an Src homology 2-dependent manner, resulting in ubiquitin-mediated degradation of Mal. This Socs1-mediated turnover of Mal has been associated with the inhibition of p65 serine phosphorylation (36). Alternatively, Socs3 has been shown to bind TNFR-associated factor 6 (TRAF6) and inhibit its activity downstream of the IL-1R (37). The IKK complex is activated by TRAF6-mediated K-63–linked ubiquitylation and Tak1-mediated phosphorylation of IKKγ. Although we demonstrated that Tak1 activation by LPS is not affected by the presence of MSP, MSP induction of Socs3 could inhibit IKK activation by interfering with TRAF6 activity in a Socs-3–dependent manner.

The PG, 15-deoxy- 12,14-PG J2 (PGJ2), an arachidonic acid-derived endogenous electrophile, is a potent inhibitor of NF-κB signaling. PGJ2 is a cyclopentenone PG that covalently modifies key Cys thiols in select proteins. Previous studies demonstrated that PGJ2 directly inhibits IKKβ activity by forming an adduct with Cys-179 in IKKβ (19). At greater concentrations, PGJ2 serves as an endogenous ligand for peroxisomal proliferator-activated receptor γ (PPARγ). PPARγ plays a central role in the regulation of NF-κB signaling through the ligand-dependent transrepression of NF-κB (38). Our preliminary data indicate that overnight treatment of macrophages with MSP induces time-dependent

production of PGJ2 (S. Yu, unpublished observations). We are currently testing the hypothesis that MSP induces expression of genes that promote the production of PGJ2, thus inhibiting the subsequent induction of IKK activity by LPS and/or promoting transrepression of NF-κB through activation of PPARγ.

We have demonstrated in this study that MSP inhibits the NF-κB–dependent upregulation of the IκB family member, IκBζ. The upregulation of IκBζ has been implicated in the positive regulation of a "late wave" of LPS-induced gene expression, including the expression of IL-6 and IL-12p40, by binding to p50 homodimers on the promoters of these genes and promoting their transcriptional activation (28, 29). The inhibition of IκBζ by MSP in primary macrophages suggests that Ron could inhibit IL-12p40 expression both directly through regulation of IKK and indirectly through the regulation of IκBζ. However, the fact that MSP inhibits IL-12p40, but not IL-6, production by LPS-activated macrophages, both targets of IκBζ, indicates that the effects of MSP on LPS-dependent gene expression mediated by its regulation of IκBζ are likely to be context dependent.

In conclusion, results presented here demonstrate that the RON receptor tyrosine kinase plays an important role in shaping the repertoire of LPS-dependent responses primarily by targeting IKK activation downstream of TLR4. The central role of the RON receptor in protecting animals from septic shock, limiting Th1-mediated inflammation, and promoting immunity to Listeria monocytogenes (8, 13, 39) suggests that this reshaping of macrophage activation by Ron is important in maintaining a critical balance between the ability of the innate immune system to promote immunity to infection, whereas at the same time protecting the host from tissue-damaging inflammation. Thus, targeting Ron or its downstream signaling components could be therapeutic in treating a large number of chronic inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Gordon S. Alternative activation of macrophages. Nat. Rev. Immunol. 2003; 3:23–35. [PubMed: 12511873]

2. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat. Rev. Immunol. 2008; 8:958–969. [PubMed: 19029990]

3. Correll PH, Morrison AC, Lutz MA. Receptor tyrosine kinases and the regulation of macrophage activation. J. Leukoc. Biol. 2004; 75:731–737. [PubMed: 14726496]

4. Lemke G, Rothlin CV. Immunobiology of the TAM receptors. Nat. Rev. Immunol. 2008; 8:327–336. [PubMed: 18421305]

5. Chen YQ, Fisher JH, Wang MH. 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. 1998; 161:4950–4959. [PubMed: 9794431]

6. Liu QP, Fruit K, Ward J, Correll PH. Negative regulation of macrophage activation in response to IFN-gamma and lipopolysaccharide by the STK/RON receptor tyrosine kinase. J. Immunol. 1999; 163:6606–6613. [PubMed: 10586055]

7. Morrison AC, Correll PH. Activation of the stem cell-derived tyrosine kinase/RON receptor tyrosine kinase by macrophage-stimulating protein results in the induction of arginase activity in murine peritoneal macrophages. J. Immunol. 2002; 168:853–860. [PubMed: 11777982]

8. Correll PH, Iwama A, Tondat S, Mayrhofer G, Suda T, Bernstein A. Deregulated inflammatory response in mice lacking the STK/RON receptor tyrosine kinase. Genes Funct. 1997; 1:69–83. [PubMed: 9680329]

9. Waltz SE, Eaton L, Toney-Earley K, Hess KA, Peace BE, Ihlendorf JR, Wang MH, Kaestner KH, Degen SJ. Ron-mediated cytoplasmic signaling is dispensable for viability but is required to limit inflammatory responses. J. Clin. Invest. 2001; 108:567–576. [PubMed: 11518730]

10. Tsutsui S, Noorbakhsh F, Sullivan A, Henderson AJ, Warren K, Toney-Earley K, Waltz SE, Power C. RON-regulated innate immunity is protective in an animal model of multiple sclerosis. Ann. Neurol. 2005; 57:883–895. [PubMed: 15929040]

11. Lentsch AB, Pathrose P, Kader S, Kuboki S, Collins MH, Waltz SE. The RON receptor tyrosine kinase regulates acute lung injury and suppresses nuclear factor kappaB activation. Shock. 2007; 27:274–280. [PubMed: 17304108]

12. Morrison AC, Wilson CB, Ray M, Correll PH. Macrophage-stimulating protein, the ligand for the stem cell-derived tyrosine kinase/RON receptor tyrosine kinase, inhibits IL-12 production by primary peritoneal macrophages stimulated with IFN-gamma and lipopolysaccharide. J. Immunol. 2004; 172:1825–1832. [PubMed: 14734766]

13. Wilson CB, Ray M, Lutz M, Sharda D, Xu J, Hankey PA. The RON receptor tyrosine kinase regulates IFN-gamma production and responses in innate immunity. J. Immunol. 2008; 181:2303–2310. [PubMed: 18684919]

14. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat. Rev. Immunol. 2003; 3:133–146. [PubMed: 12563297]

15. Lee ES, Kalantari P, Tsutsui Section S, Klatt A, Holden J, Correll PH, Power Section C, Henderson AJ. RON receptor tyrosine kinase, a negative regulator of inflammation, inhibits HIV-1 transcription in monocytes/ macrophages and is decreased in brain tissue from patients with AIDS. J. Immunol. 2004; 173:6864–6872. [PubMed: 15557181]

16. Nikolaidis N, Gray J, Gurusamy D, Fox W, Stuart W, Huber N, Waltz S. RON receptor tyrosine kinase negatively regulates TNFalpha production in alveolar macrophages by inhibiting NF-kappaB activity and ADAM17 production. Shock. 2010; 33:197–204. [PubMed: 19487969]

17. Lutz MA, Correll PH. Activation of CR3-mediated phagocytosis by MSP requires the RON receptor, tyrosine kinase activity, phosphatidylinositol 3-kinase, and protein kinase C zeta. J. Leukoc. Biol. 2003; 73:802–814. [PubMed: 12773513]

18. Prabhu KS, Arner RJ, Vunta H, Reddy CC. Up-regulation of human myo-inositol oxygenase by hyperosmotic stress in renal proximal tubular epithelial cells. J. Biol. Chem. 2005; 280:19895–19901. [PubMed: 15778219]

19. Vunta H, Davis F, Palempalli UD, Bhat D, Arner RJ, Thompson JT, Peterson DG, Reddy CC, Prabhu KS. The anti-inflammatory effects of selenium are mediated through 15-deoxy-Delta12,14-prostaglandin J2 in macrophages. J. Biol. Chem. 2007; 282:17964–17973. [PubMed: 17439952]

20. Teal HE, Ni S, Xu J, Finkelstein LD, Cheng AM, Paulson RF, Feng GS, Correll PH. GRB2-mediated recruitment of GAB2, but not GAB1, to SF-STK supports the expansion of Friend virus-infected erythroid progenitor cells. Oncogene. 2006; 25:2433–2443. [PubMed: 16314834]

21. Thomas KE, Galligan CL, Newman RD, Fish EN, Vogel SN. Contribution of interferon-beta to the murine macrophage response to the toll-like receptor 4 agonist, lipopolysaccharide. J. Biol. Chem. 2006; 281:31119–31130. [PubMed: 16912041]

22. Saito S, Matsuura M, Hirai Y. Regulation of lipopolysaccharide-induced interleukin-12 production by activation of repressor element GA-12 through hyperactivation of the ERK pathway. Clin. Vaccine Immunol. 2006; 13:876– 883. [PubMed: 16893987]

23. Wei X, Hao L, Ni S, Liu Q, Xu J, Correll PH. Altered exon usage in the juxtamembrane domain of mouse and human RON regulates receptor activity and signaling specificity. J. Biol. Chem. 2005; 280:40241–40251. [PubMed: 16166096]

24. Wei X, Ni S, Correll PH. Uncoupling ligand-dependent and -independent mechanisms for mitogen-activated protein kinase activation by the murine RON receptor tyrosine kinase. J. Biol. Chem. 2005; 280:35098–35107. [PubMed: 16103119]

25. Buss H, Dö rrie A, Schmitz M, Hoffmann E, Resch K, Kracht M. Constitutive and interleukin-1-inducible phosphorylation of p65 NF-kappaB at serine 536 is mediated by multiple protein kinases including IkappaB kinase (IKK)-alpha, IKKbeta, IKKepsilon, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription. J. Biol. Chem. 2004; 279:55633–55643. [PubMed: 15489227]

26. Chen LF, Greene WC. Shaping the nuclear action of NF-kappaB. Nat. Rev. Mol. Cell Biol. 2004; 5:392–401. [PubMed: 15122352]

27. Yamamoto M, Takeda K. Role of nuclear IkappaB proteins in the regulation of host immune responses. J. Infect. Chemother. 2008; 14:265–269. [PubMed: 18709529]

28. Yamazaki S, Muta T, Takeshige K. A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. J. Biol. Chem. 2001; 276:27657–27662. [PubMed: 11356851]

29. Yamamoto M, Yamazaki S, Uematsu S, Sato S, Hemmi H, Hoshino K, Kaisho T, Kuwata H, Takeuchi O, Takeshige K, et al. Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. Nature. 2004; 430:218–222. [PubMed: 15241416]

30. Wetzel CC, Leonis MA, Dent A, Olson MA, Longmeier AM, Ney PA, Boivin GP, Kader SA, Caldwell CC, Degen SJ, Waltz SE. Short-form RON receptor is required for normal IFN-gamma production in concanavalin A-induced acute liver injury. Am. J. Physiol. Gastrointest. Liver Physiol. 2007; 292:G253–G261.

31. Narasimhan M, Ammanamanchi S. Curcumin blocks RON tyrosine kinase-mediated invasion of breast carcinoma cells. Cancer Res. 2008; 68:5185–5192. [PubMed: 18593918]

32. Zhou YQ, Chen YQ, Fisher JH, Wang MH. Activation of the RON receptor tyrosine kinase by macrophage-stimulating protein inhibits inducible cyclooxygenase-2 expression in murine macrophages. J. Biol. Chem. 2002; 277:38104–38110. [PubMed: 12177064]

33. Ni S, Zhao C, Feng GS, Paulson RF, Correll PH. A novel Stat3 binding motif in Gab2 mediates transformation of primary hematopoietic cells by the Stk/RON receptor tyrosine kinase in response to Friend virus infection. Mol. Cell. Biol. 2007; 27:3708–3715. [PubMed: 17353274]

34. El Kasmi KC, Smith AM, Williams L, Neale G, Panopoulos AD, Panopolous, Watowich SS, Häcker H, Foxwell BM, Murray PJ. Cutting edge: a transcriptional repressor and corepressor induced by the STAT3-regulated anti-inflammatory signaling pathway. J. Immunol. 2007; 179:7215–7219. [PubMed: 18025162]

35. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. Nat. Rev. Immunol. 2007; 7:454–465. [PubMed: 17525754]

36. Mansell A, Smith R, Doyle SL, Gray P, Fenner JE, Crack PJ, Nicholson SE, Hilton DJ, O'Neill LA, Hertzog PJ. Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. Nat. Immunol. 2006; 7:148–155. [PubMed: 16415872]

37. Frobøse H, Rønn SG, Heding PE, Mendoza H, Cohen P, Mandrup-Poulsen T, Billestrup N. Suppressor of cytokine Signaling-3 inhibits interleukin-1 signaling by targeting the TRAF-6/TAK1 complex. Mol. Endocrinol. 2006; 20:1587–1596. [PubMed: 16543409]

38. Straus DS, Glass CK. Anti-inflammatory actions of PPAR ligands: new insights on cellular and molecular mechanisms. Trends Immunol. 2007; 28:551–558. [PubMed: 17981503]

39. Lutz MA, Gervais F, Bernstein A, Hattel AL, Correll PH. STK receptor tyrosine kinase regulates susceptibility to infection with Listeria monocytogenes. Infect. Immun. 2002; 70:416–418. [PubMed: 11748211]

Abbreviations used in this paper

HA	hemagglutinin
HEK293	human embryonic kidney 293
IKK	IκB kinase
iNOS	inducible NO synthase
MSP	macrophage-stimulating protein
PGJ2	15-deoxy- 12,14-PGJ2
PPAR	peroxisomal proliferator-activated receptor
Socs	suppressor of cytokine signaling
Tak1	TGF-β–activated kinase
TRAF	TNFR-associated factor
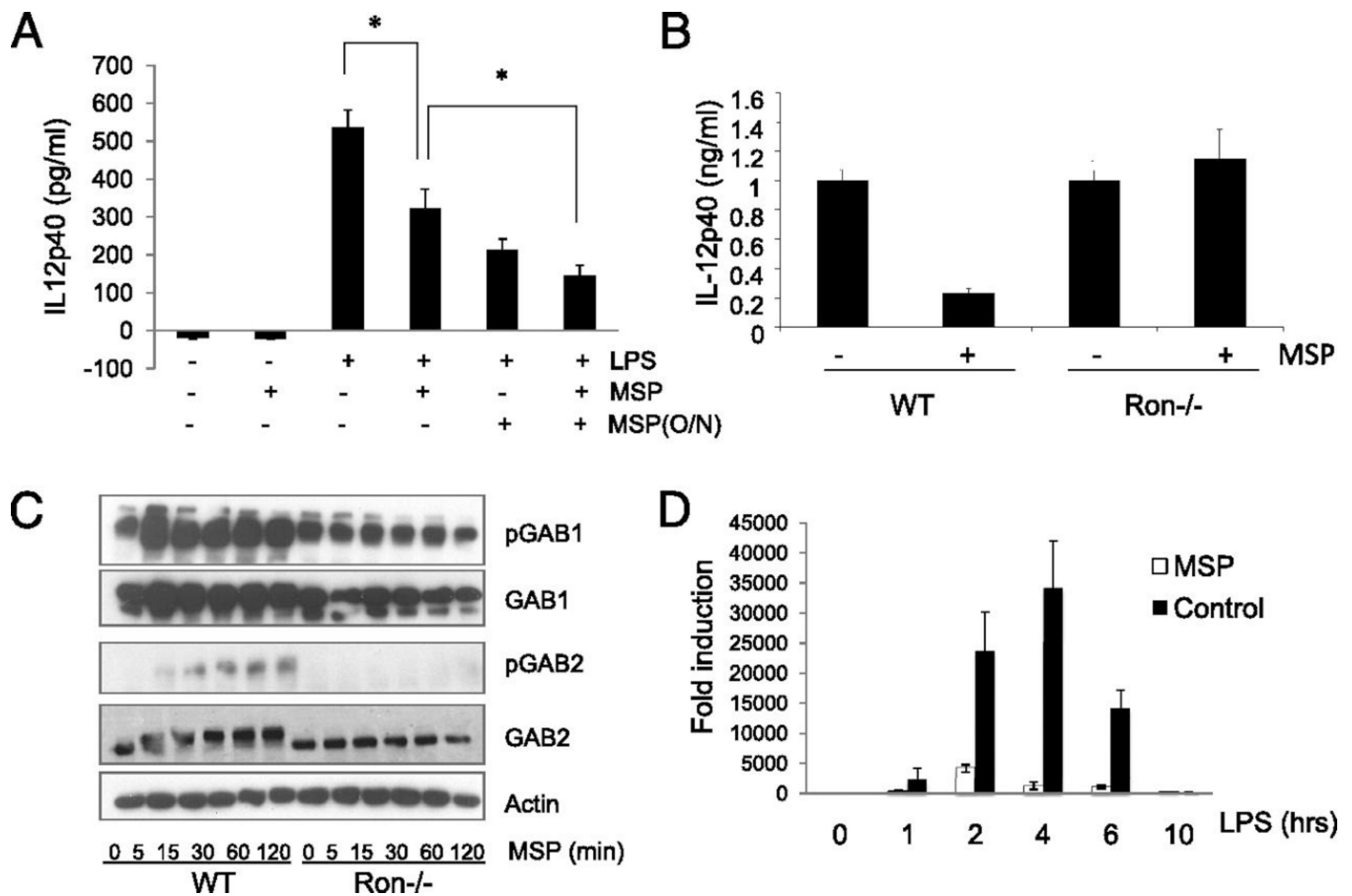
Figure 1. MSP stimulation of the RON receptor inhibits IL-12p40 production in primary macrophages

A, Primary peritoneal macrophages were cultured overnight in the presence or absence of 100 ng/ml MSP followed by stimulation with LPS with or without MSP. IL-12p40 production was assessed by ELISA. *p < 0.01. B, Primary resident peritoneal macrophages were collected from wild-type and Ron–/– BALB/c mice by lavage. Macrophages were isolated by adherence to plastic. The cells were stimulated overnight with 100 ng/ml MSP followed by stimulation with 100 ng/ml LPS for 24 h, and supernatants were collected. IL-12p40 production was assessed by ELISA. C, Primary peritoneal macrophages were isolated from wild-type and Ron–/– mice by lavage, and macrophages were isolated by adherence to plastic. After overnight culture, the cells were stimulated with MSP for the indicated times, and phosphorylation of Gab1 and Gab2 was assessed by Western blot analysis. D, Peritoneal macrophages were stimulated with 100 ng/ml MSP overnight followed by activation with 100 ng/ml LPS for the indicated times. IL-12p40 mRNA expression was assessed by real-time PCR.
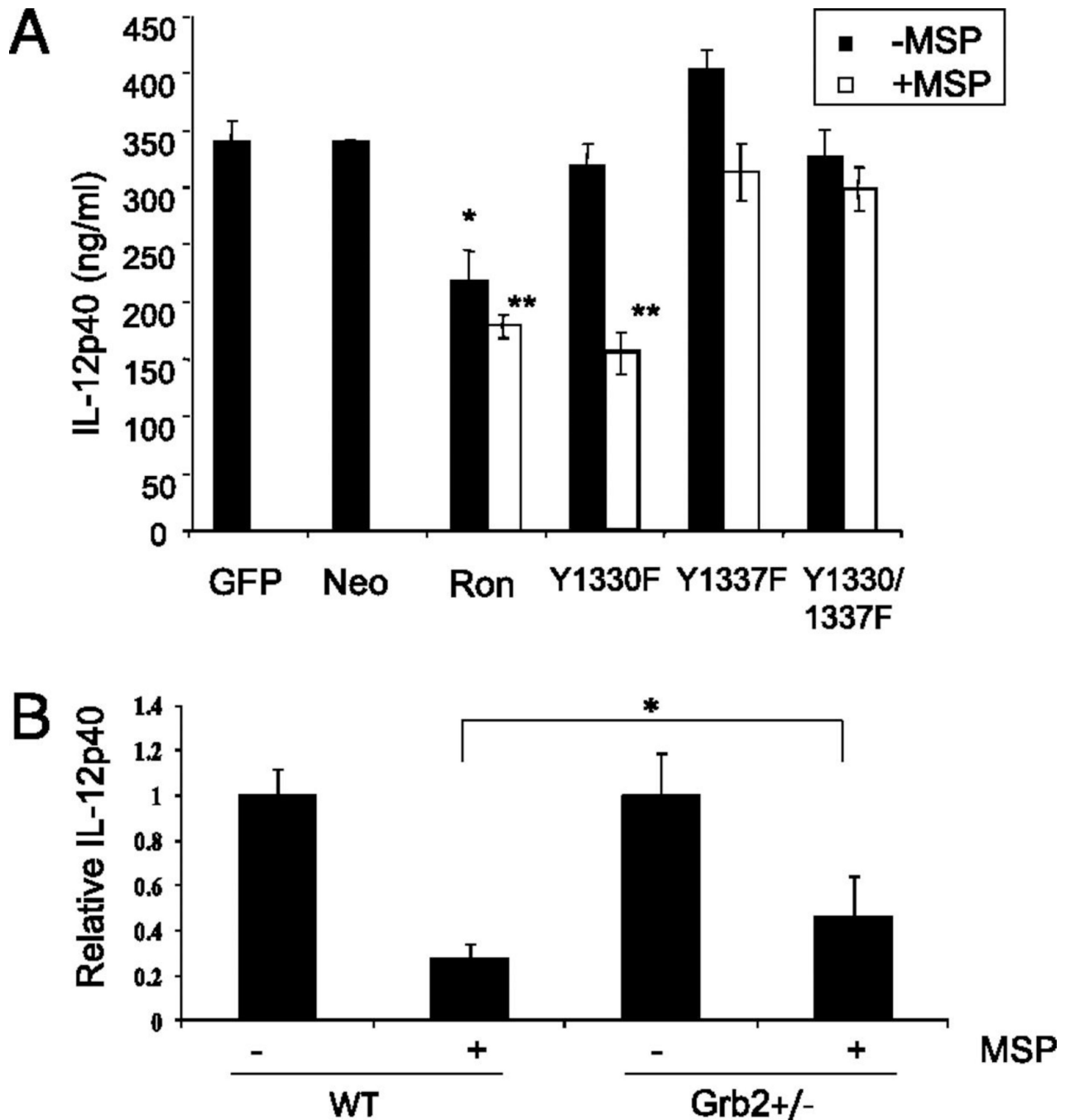
Figure 2. Tyrosines in the C-terminal tail of Ron mediate the inhibition of LPS-induced IL-12p40 production in primary macrophages

A, Bone marrow-derived macrophages were isolated from primary murine bone marrow by adherence to plastic. The cells were cultured in L929 cell conditioned media and infected with murine stem cell virus-based retroviral vectors harboring wild-type or mutant forms of Ron. The cells were then treated overnight with 100 ng/ml MSP followed by stimulation with 100 ng/ml LPS for 24 h. Supernatants were collected, and IL-12p40 levels were assessed by ELISA. B, Resident peritoneal macrophages from wild-type or Grb2+/− animals were cultured in the presence or absence of MSP for 12 h followed by stimulation with 100

ng/ml LPS for 24 h. IL-12p40 levels in the supernatants were assessed by ELISA. *p < 0.05; **p < 0.01.
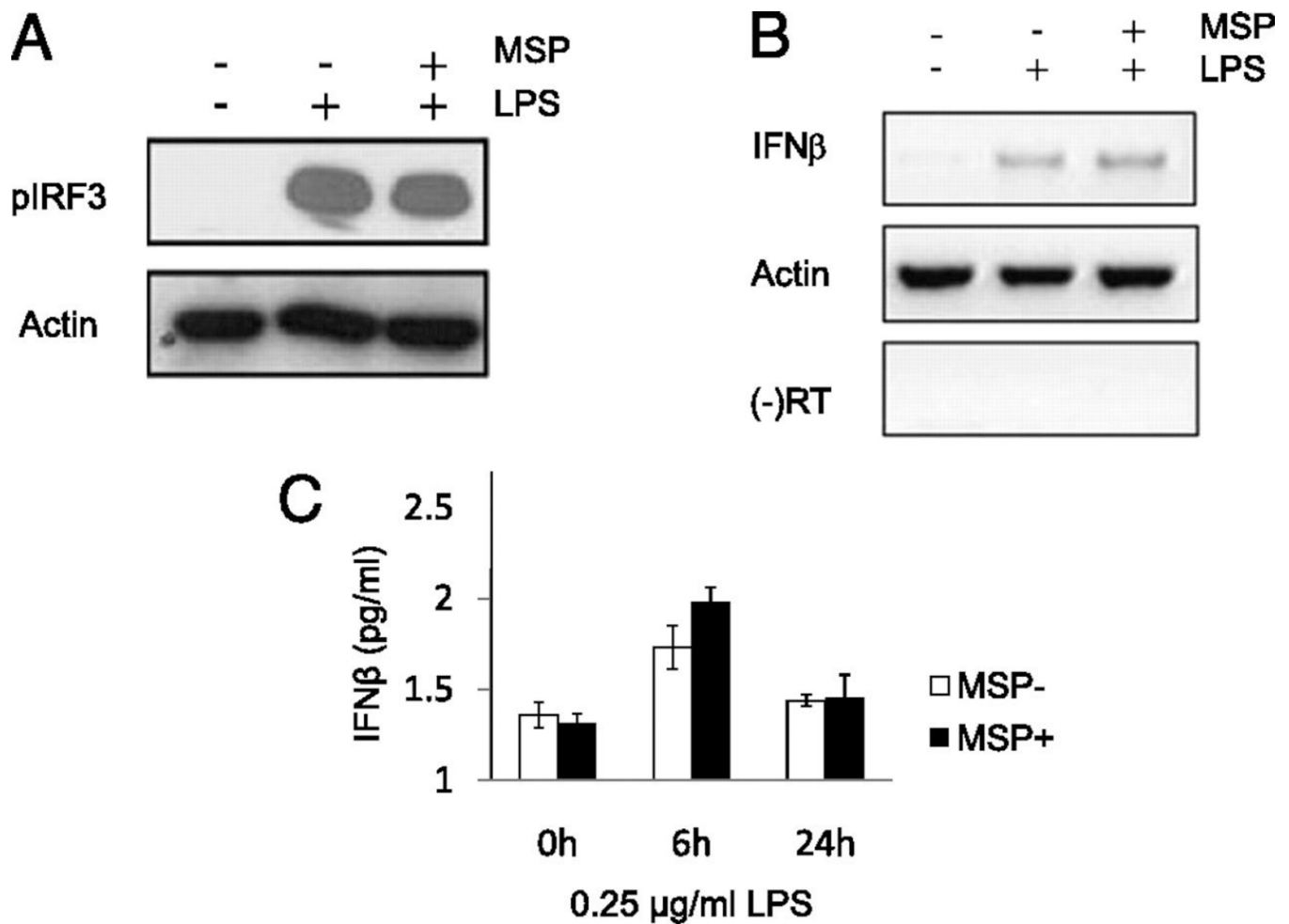
Figure 3. MSP does not inhibit MyD88-independent signaling by TLR4
A, Primary resident peritoneal macrophages were isolated by lavage and adherence to plastic. The cells were treated with 100 ng/ml MSP overnight followed by stimulation with 100 ng/ml LPS. Cell lysates were prepared and IRF3 phosphorylation was assessed by Western blot analysis with a phosphospecific IRF3 Ab. The blot was stripped and reprobed with anti-IRF3. B, Primary resident peritoneal macrophages were pretreated with MSP and stimulated with 100 ng/ml LPS for 6 h. RNA was extracted and expression of IFN-b was assessed by RT-PCR. C, Primary resident peritoneal macrophages were pretreated with 100 ng/ml MSP overnight and then stimulated with 250 ng/ml LPS for the indicated times. Cell culture supernatants were collected at the indicated times, and IFN-b production was assessed by ELISA.
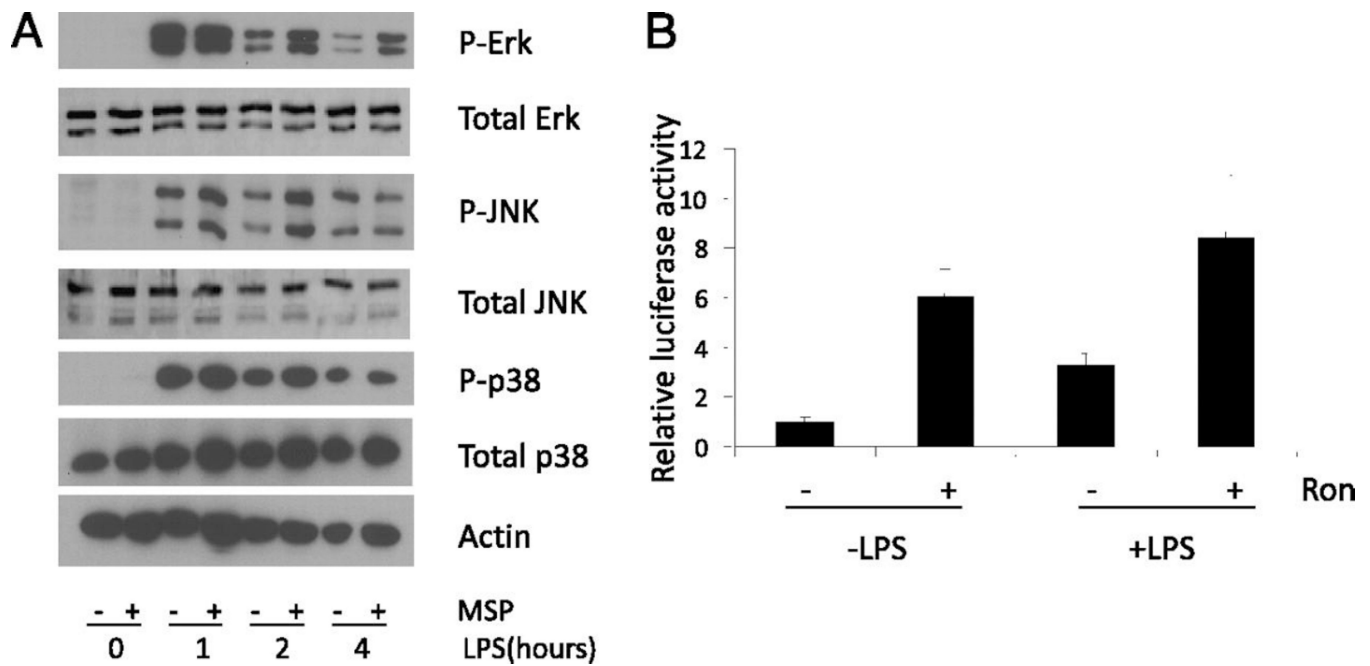
Figure 4. MSP enhances MAPK signaling and AP-1 activity in LPS-stimulated cells
A, Primary resident peritoneal macrophages were stimulated with 100 ng/ml overnight followed by 100 ng/ml LPS. Cell lysates were extracted at the indicated times, and phosphorylation of ERK, p38, and JNKwere assessed by Western blot analysis with phosphospecific Abs. Blots were stripped and reprobed with anti-ERK, anti-p38, and anti-JNK. B, 293 cells stably transfected with TLR4 and MD-2 were transfected with pcDNA3.1 empty vector or pcDNA3.1Ron-HA, a CD14 expression vector, and anAP-1 luciferase reporter construct. After an overnight transfection, the cells were treated with or without 100 ng/ml LPS for 4 h and luciferase activity was measured.
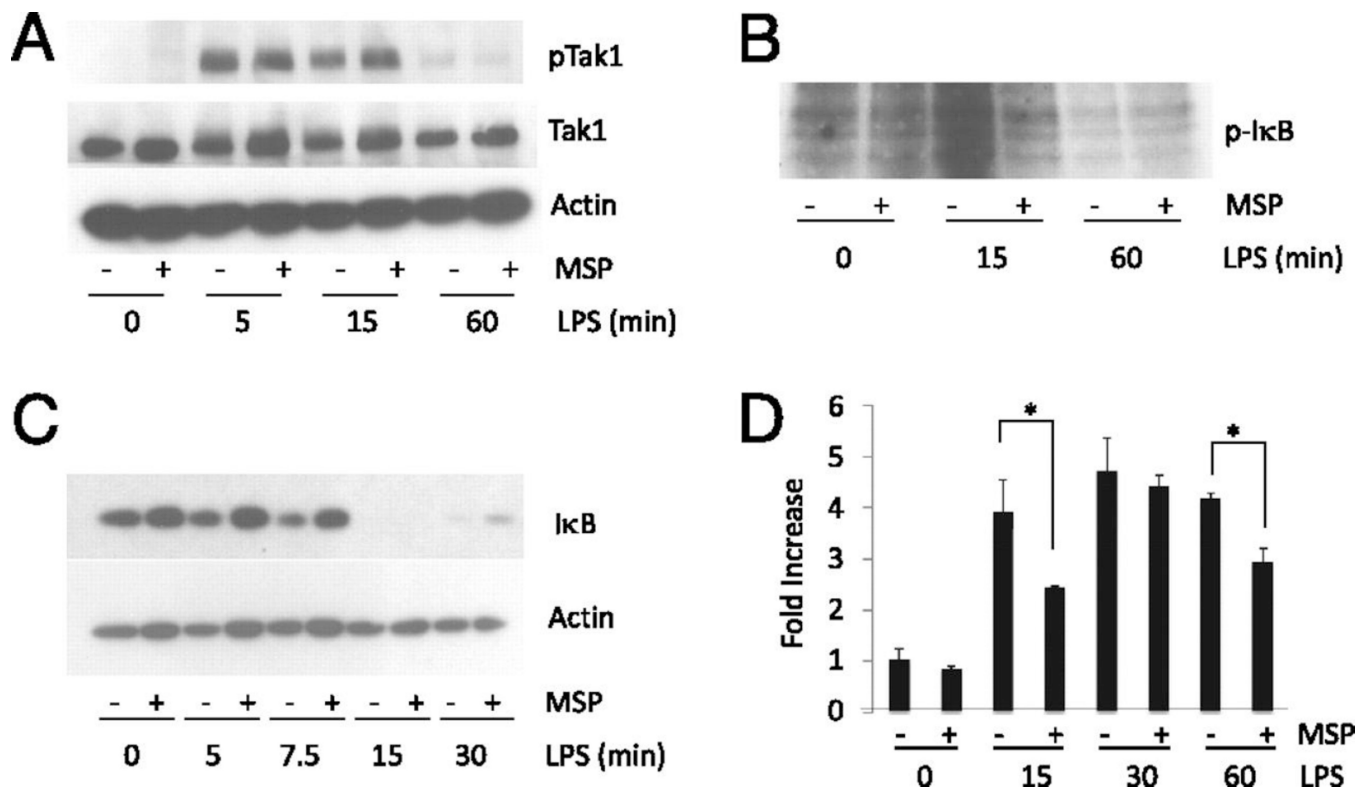
Figure 5. MSP delays IKK activity, IκB degradation, and NF-κB binding after LPS stimulation
A, Resident peritoneal macrophages were stimulated with MSP overnight followed by stimulation with 100 ng/ml LPS for the indicated times. Tak1 phosphorylation was assessed by Western blot analysis using a phospho-specific Tak1 Ab. B, Thioglycolate-elicited macrophages were isolated by adherence and stimulated with 100 ng/ml MSP overnight followed by 100 ng/ml LPS. At the indicated times, cell lysates were extracted and IKK activity was measured by in vitro kinase assay using IκB as a substrate. C, Primary resident peritoneal macrophages were stimulated overnight with 100 ng/ml MSP followed by 100 ng/ml LPS. Cell lysates were extracted at the indicated times, and IκBα levels were assessed by Western blot analysis. The blots were stripped and reprobed with anti-actin. D, Thioglycolate-elicited macrophages were isolated by adherence to plastic and stimulated with 100 ng/ml MSP overnight followed by 100 ng/ml LPS. Nuclear extracts were isolated at the indicated times, and binding to DNA was assessed by ELISA. *p < 0.01.

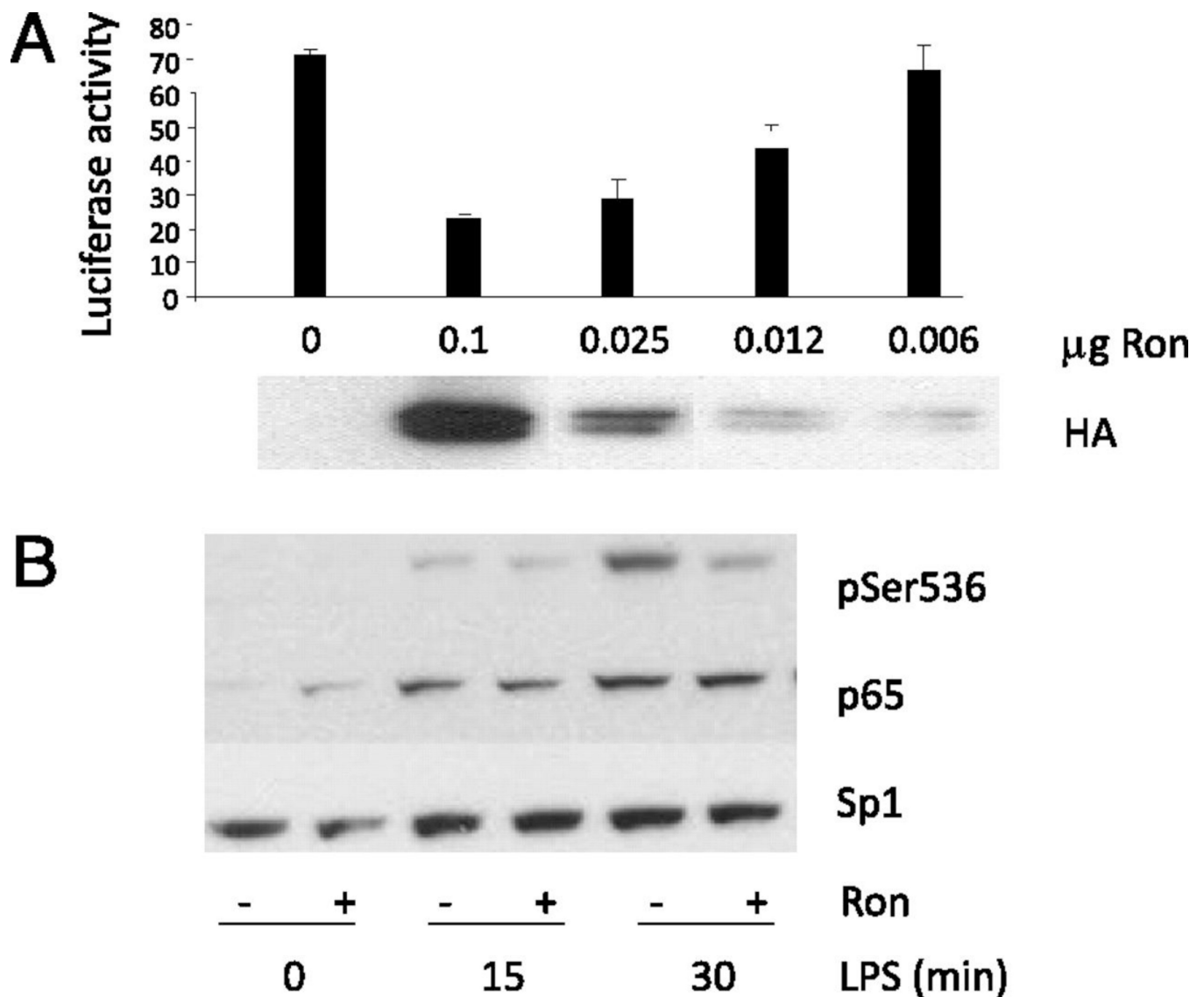**Figure 6. Ron inhibits NF-kB transcriptional activity and p65 serine phosphorylation in response to LPS**

A, HEK293 cells expressing TLR4 and MD-2 were cotransfected with the same empty vector or HA-tagged Ron and an NF-kB luciferase reporter. The transfected cells were stimulated with 100 ng/ml LPS and luciferase activity was assessed. Lysates were also analyzed by Western blot using an anti-HA Ab. B, HEK293 cells expressing TLR4 and MD-2 were transfected with or without Ron and stimulated with 100 ng/ml LPS. Nuclear lysates were isolated and phosphorylation of p65 at serine 536 was assessed by Western blot analysis using a phosphospecific Ab. Blots were stripped and reprobed with antip65 and anti-Sp1.
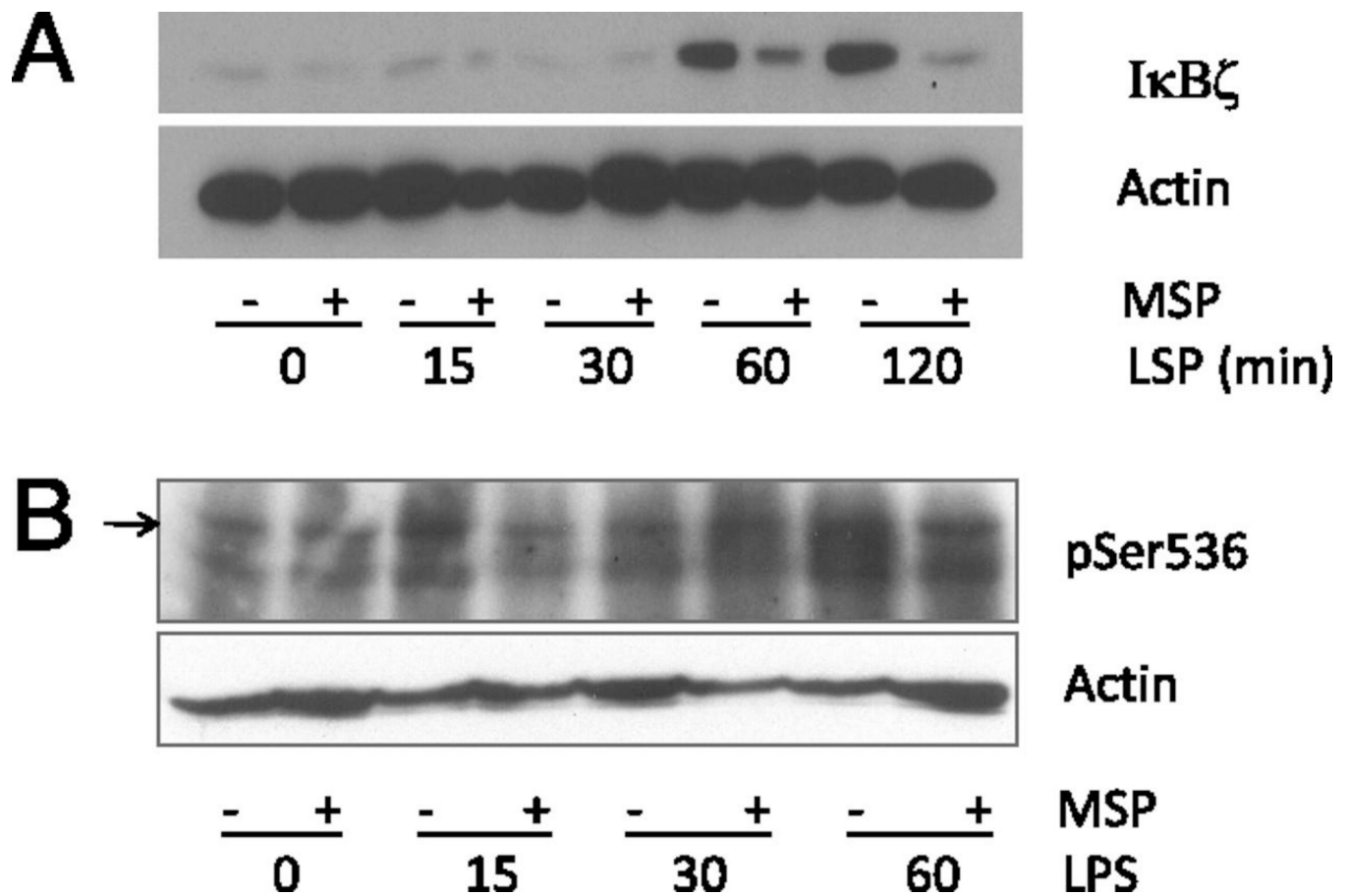
Figure 7. MSP inhibits NF-kB transcriptional activity and delays serine phosphorylation of p65 in primary macrophages

A, Primary resident peritoneal macrophages were harvested by lavage and isolated by adherence to plastic. The cells were stimulated with 100 ng/ml MPS overnight followed by 100 ng/ml LPS. Cell lysates were harvested at the indicated times, and IkBz expression was assessed by Western blot analysis. The blot was stripped and reprobed with anti-actin. B, Primary resident peritoneal macrophages were stimulated with LPS for the indicated times in the presence or absence of MSP. Serine phosphorylation of p65 was assessed by Western blot analysis.
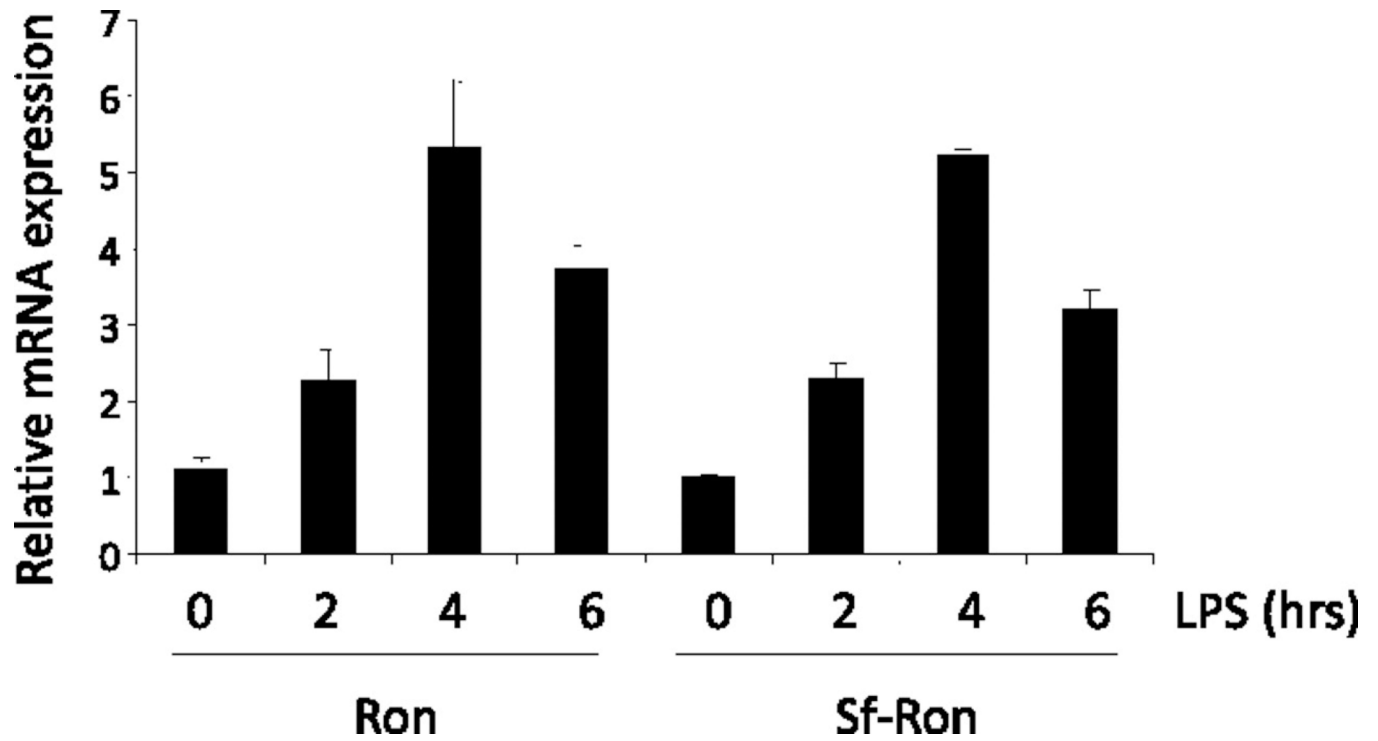
Figure 8. LPS induces expression of Ron and Sf-Ron in primary macrophages
Primary resident peritoneal macrophages were stimulated for the indicated times with 100 ng/ml LPS. Expression of Ron and Sf-Ron were assessed by real-time PCR. $p < 0.001$ for all values relative to the zero time point for each gene.