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The RON Receptor Tyrosine Kinase Regulates IFN-γ Production and Responses in Innate Immunity

Caleph B. Wilson,1* Manujendra Ray,1† Michael Lutz,* Daniel Sharda,* Jie Xu,* and Pamela A. Hankey2*†

Receptor tyrosine kinases are emerging as a class of key regulators of innate immune responses. We have shown previously that the RON receptor tyrosine kinases (murine Stk), expressed on tissue-resident macrophages, inhibit classical macrophage activation while promoting hallmarks of alternative activation, thus regulating the critical balance between the inflammatory and wound-healing properties of activated macrophages. We have also shown previously that RON−/− mice are more susceptible to in vivo endotoxin challenge than wild-type mice, suggesting that the expression of this receptor confers a degree of endotoxin resistance to these animals. Here we demonstrate that, in response to in vivo LPS challenge, RON knockout mice indicates that the enhanced susceptibility of RON−/− mice to endotoxin challenge is dependent on IFN-γ-mediated signals. In vitro studies demonstrate that stimulation of primary peritoneal macrophages with macrophage-stimulating protein, the ligand for RON, inhibits IFN-γ-induced STAT1 phosphorylation and CIITA expression, resulting in reduced surface levels of MHC class II. Further studies demonstrating the induction of suppressor of cytokine signaling 1 via macrophage-stimulating protein/RON signaling provide a potential mechanistic insight into this regulatory pathway. These results indicate that the RON receptor regulates both the production of and response to IFN-γ, resulting in enhanced susceptibility to endotoxin challenge. The Journal of Immunology, 2008, 181: 2303–2310.

The innate immune system serves as the body’s first line of defense against invading pathogens. Armored with evolutionarily conserved receptors including membrane-spanning TLRs and intracellular NOD receptors, capable of recognizing a wide range of pathogen-associated molecular patterns, macrophages are poised to respond to invading microbes (1, 2). LPS, a component of the outer envelope of Gram-negative bacteria, is the prototypic TLR-4 ligand (3, 4). The activation of TLR-4 by LPS stimulates macrophages to produce a vast array of proinflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-12 as well as some anti-inflammatory cytokines like IL-10 (5). Whereas the recognition of LPS activates macrophages, IFN-γ produced by IL-12-responsive immune cells, including NK, NK T cells (NKT),3 and innate T cells (6, 7), acts in a paracrine-positive feedback loop to sustain and further bolster macrophage activation (7–9), ultimately leading to the more efficient clearance of LPS-bearing pathogens (9).

The increased resistance of IFN-γ receptor knockout mice to LPS challenge illustrates a central role for IFN-γ-mediated signaling in LPS induced inflammatory responses (10). However, although innate immune signals play a critical role in mounting an effective response to invading pathogens, these signals must be tightly regulated to prevent excessive or chronic inflammation and disease. Suppressor of cytokine signaling (SOCS) 1 and SOCS3, members of the suppressor of cytokine signaling and cytokine-inducible SH2 domain-containing protein family, have been found to down-regulate both TLR and IFN signaling (11–13), resulting in protection from septic shock.

Recently, Rothlin et al. (14) demonstrated that tyrosine-based activation motif (TAM) receptors can inhibit TLR signaling by inducing SOCS1 and SOCS3 in a STAT1-dependent manner. Tyro3, Axl, and Mer (TAM receptors) are a phylogenetically related group of receptor tyrosine kinases expressed on cells of the innate immune system, including macrophages and dendritic cells. Tyro3, Axl, and Mer triple-knockout mice show diffuse immune activation and develop a widespread lymphoproliferative autoimmune disease. Dendritic cells from these mice are hyperresponsive to TLR-mediated activation (14, 15). The Axl tyrosine kinase has been shown to be responsible for the IFN-α-mediated inhibition of FcyR-induced TNF-α production, by inducing Twist proteins, the E box-binding NF-κB transcriptional repressors (16). Mer single-knockout mice are more susceptible to in vivo endotoxin challenge, and macrophages from these mice produce higher levels of TNF-α as a result of increased endotoxin induced NF-κB activity. Mer−/− macrophages also show a decreased capacity to clear apoptotic cells (15, 17).

The RON receptor, a 180-kDa receptor tyrosine kinase, is also a negative regulator of immune responses by virtue of its expression on tissue-resident macrophages (18, 19). RON−/− mice are more susceptible to in vivo endotoxin challenge (19), and signaling...
through this receptor down-regulates LPS- and IFN-γ-induced COX-2 expression (20) and NO production (21). Furthermore, by inhibiting inducible NO synthase (iNOS) expression and inducing arginase activity in macrophages, RON diverts the common metabolic substrate l-arginine from the production of NO to the production of ornithine, an intermediate in the collagen synthesis pathway (22). In doing so, signaling through RON down-regulates the classical activation of macrophages, which is associated with inflammation, and skews them toward a more alternatively activated phenotype that is associated with wound healing (23).

Macrophage-stimulating protein (MSP), the ligand for RON, is an 80-kDa serum protein that belongs to a family of highly conserved kringle domain containing proteins including plasminogen and hepatocyte growth factor. It is secreted mainly by the liver in an inactive pro-MSP form and is cleaved into its active form by serum proteases of the intrinsic coagulation cascade at sites of inflammation (24, 25). MSP has been shown to enhance complement mediated phagocytosis, induce macrophage arginase activity, and induce shape change in peritoneal macrophages (22, 26, 27).

Previously, we showed that treatment of peritoneal macrophages with MSP significantly down-regulates the production of LPS- and IFN-γ-induced IL-12p70 by down-regulating the p40 subunit of this heterodimeric cytokine (18).

We show here that RON regulates IL-12p40 expression in vivo following endotoxin challenge, resulting in elevated serum levels of IL-12p70 and IFN-γ. Furthermore, we provide evidence that the increased susceptibility of RON+/− mice to septic shock is dependent on signaling through the IFN-γ receptor. Finally, we demonstrate that MSP negatively regulates the responsiveness of macrophages to IFN-γ stimulation, associated with an increase in SOCS1 and SOCS3 expression. Taken together, our data indicate that RON serves as a critical checkpoint in regulating endotoxin-induced innate immune responses by regulating the production of, and response to, IFN-γ.

Materials and Methods

Cells and animals

The following mouse strains were used: wild-type CD-1 mice; CD-1 mice with a targeted mutation in the RON gene (RON+/−); wild-type C57BL/6 (The Jackson Laboratory); and RON+/− C57BL/6 mice (generated by backcrossing the original targeted mutation in the RON gene for 12 generations onto this background); and wild-type BALB/c mice. RON+/− × IFN-γR−/− (doubled knock-out [DKO]) mice were generated by crossing RON−/− mice on the C57BL/6 background with IFN-γR−/− mice on a C57BL/6 background. Murine resident peritoneal macrophages were harvested and cultured as previously described (22). 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); recombinant human MSP (R&D Systems); recombinant human IFN-γ, IL-12 (rHuIFN-γ, rHuIL-12; Genzyme); IFN-α (Genzyme); IFN-β (Genzyme); murine IL-12p70 (Genzyme). IFN-α was purified by affinity chromatography (28) and was used at a concentration of 2,000 U/ml. IL-12 was used at a concentration of 10 ng/ml. Reagents and probes were designed by the Nucleic Acid Facility at The Pennsylvania State University, College of Medicine (University Park, PA). Primers and probes were purchased from Applied Biosystems (Foster City, CA) and Life Technologies (Carlsbad, CA). Primers and probes were designed to be specific to the mouse gene of interest and would not cross-react with other species. Primers and probes were designed to be specific to the mouse gene of interest and would not cross-react with other species. Primers and probes were designed to be specific to the mouse gene of interest and would not cross-react with other species.

Flow cytometry

Wild-type and RON+/− peritoneal macrophages were treated as indicated. Flow cytometric analysis was performed as previously described. The cell surface expression of MHC class II and IFN-γR was determined by staining with fluorescent-labeled Abs against mouse MHC class II (I-A<sup>+</sup>) and CD119, respectively. The stained cells were then analyzed on a Beckman Coulter XL flow cytometer.

Splenocytes from un.injected and LPS-injected mice were collected and plated for 3 h in complete cell culture media (supplemented with 1-glutamine, nonessential amino acids, sodium pyruvate, ciprofloxacin, and 10% FBS) with 5 μg/ml brefeldin A. Alternatively, splenocytes were cultured overnight in complete medium supplemented with 5 ng/ml rmIL-12, 10 ng/ml rmIL-16, 20 ng/ml rmIL-2, and 5 μg/ml brefeldin A. The cultured cells were collected and subsequently stained with anti-NC1.1 PE and anti-CD3 FITC. After extracellular staining, the cells were fixed in 2% paraformaldehyde for 10 min at room temperature. The fixed cells were then permeabilized with saponin buffer (0.1% BSA, 0.1% saponin) and stained with anti-IFN-γ PE-CY7. Flow cytometric analysis was performed on a Beckman Coulter FC500 flow cytometer.

Cell sorting for gene expression studies

Splenocytes were collected from wild-type and RON−/− mice and depleted of RBCs using ammonium chloride-potassium lysis buffer. Splenocytes were stained with anti-NK1.1 PE and anti-CD3 FITC. NK1.1<−CD3− cells were sorted using a Cytomix Influx cell sorter. RNA was collected from the sorted cells using the RNeasy kit from Qiagen.

RNA extraction, real-time RT-PCR, and RT-PCR

RNA was extracted from cultured or sorted cells using the RNeasy mini column kit (Qiagen). Total RNA from whole wild-type (WT) and RON−/− murine spleens was isolated using TRIzol (Invitrogen). Reverse transcription for RT-PCR was conducted for 40 min at 42°C using oligo(dT)15 primers (Applied Biosystems) and murine leukemia virus reverse transcriptase (Applied Biosystems). PCR for murine (m) CIITA (28), mIL-12 p40 (18), and m-fgp-actin (18) were conducted as previously described. PCR products were run on a 2% agarose gel. Real-time PCR was conducted on an Applied Biosystems 7300. Reverse transcription for real-time PCR experiments was conducted using the Applied Biosystems High Capacity RT Kit according to the manufacturer’s recommendations. Gene-specific primers and probes were designed by the Nucleic Acid Facility at The Pennsylvania State University using Primer Express V.1.0 (Applied Biosystems). 18S and GAPDH were used as the internal standards (GAPDH TaqMan primer and probe set was purchased from Applied Biosystems). Primer sequences and probes are as follows: mSOCS1 sense 5′-CCCTGCG GTCCGGCAAGAC-3′, mSOCS1 antisense 5′-AAGGACCTCACTGATGTC-3′, and probe 5′-6-FAM (ThGCGCGCATCTTTAAT CCC) BHQ-1-3′, mSOCS3 sense 5′-GCCACCTGGAATCTTACATGAGAA-3′, mSOCS3 antisense 5′-GGGACCATCATACTGATGACGAA-3′, and probe 5′-6-FAM (dAGTCGCTCGTGGACAACCTTGGG) BHQ-1-3′, mRON sense 5′-GCCCTCGGGAATGACCTACTCTT-3′, mRON antisense 5′-AGGAGAGATAGGAGACGACCCAGC-3′, and probe 5′-6-FAM (dAGG TCTTCAAGGGCCATCTGCCG) BHQ-1-3′.

Western blot analysis

Peritoneal macrophages from WT and RON−/− mice were treated as indicated. The cells were washed with PBS and lysed for 15 min on ice in lysis buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.5% Triton X-100), supplemented with protease and phosphatase inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, 1 mM sodium orthovanadate, and 10 μM sodium fluoride). SDS-PAGE was performed using either 10 or 12.5% bisacrylamide gels. The protein was transferred onto Immobilon P polyvinylidene difluoride membranes (Millipore), and the blots were blocked with 5% nonfat dry milk-BSA for 1 h at room temperature. The blots were then incubated overnight with primary Ab. Anti-rabbit/mouse secondary Abs conjugated with HRP were used at 1:10,000/1:8,000, respectively, for 1 h at room temperature. ECL Plus (Amersham) was used to develop the blots.

In vivo endotoxin challenge

WT and RON−/− mice were challenged with 20 mg/kg LPS i.p., IFN-γ−/− and RON−/− × IFN-γR−/− DKO mice were challenged with 20, 35, or 50 mg/kg LPS i.p. The mice were monitored every 6 h for a total of 60 h with survival recorded for each interval. WT and RON−/− CD-1 mice were injected i.p. with 20 mg t-galactosamine (t-GalN) and different doses of LPS as indicated. Serum was collected from these mice 6 h postinjection. The serum samples were sent to Ani Lyrics for analysis of aspartate aminotransferase and alanine aminotransferase levels.

Cytokine assay

Serum samples were collected from WT and RON−/− mice injected with 20 mg/kg LPS for different durations of time. The serum cytokines were
In mice, the IL-12-responsive IFN-γ in vivo following LPS challenge. A, WT and RON−/− C57BL/6 mice were challenged with 20 mg/kg LPS. Spleens were collected at 3 h postinjection, and IL12-p40 gene expression was assessed by RT-PCR (WT n = 8, RON−/− n = 8). Serum was collected from WT and RON−/− mice at indicated time points following i.p. injection with 20 mg/kg LPS. Lanes A–H and I–J represent individual mice. The levels of IL-12p70 (B) and IFN-γ (C) were assessed using a multiplex cytokine assay kit (at each time point: WT n = 8; RON−/− n = 8) *, p < 0.05.

Results

Enhanced production of IL-12 and IFN-γ in RON−/− mice following endotoxin challenge

We have shown previously that the in vitro stimulation of primary peritoneal macrophages with MSP substantially inhibits the LPS- and IFN-γ-induced production of IL-12p70, particularly the expression of the p40 subunit, and that this inhibition is mediated by the RON receptor (18). To determine whether RON regulates IL-12p40 expression in vivo, we injected WT and RON−/− mice with LPS for increasing durations of time. RNA was extracted from whole spleens at 1 h and 3 h after injection, and IL-12p40 expression was assessed by RT-PCR. At 1 h, the level of IL-12p40 in the spleens of WT and RON−/− mice was insignificant (data not shown). However, at 3 h postinjection, we saw a significant up-regulation of IL-12p40 expression in several RON−/− animals, whereas there was little or no induction of IL-12p40 in the WT animals (Fig. 1A). Congruent to the elevation of IL-12p40 seen in the spleen, we also saw higher levels of the biologically active IL12p70 in the serum of RON−/− mice at 6 h postinjection (Fig. 1B).

IL-12 produced by macrophages early in an innate immune response promotes the induction of IFN-γ by IL-12-responsive immune cells (8). It was therefore not surprising that we observed enhanced levels of IFN-γ in the bloodstream of RON−/− mice at 6 and 12 h postinjection with the differences being statistically significant at 12 h (Fig. 1C). Taken together, these studies suggest that, during the initial phase of the response to LPS, an unchecked production of IL-12p40 in RON−/− mice leads to the enhanced production of IFN-γ by IL-12-responsive cells.

Enhanced IFN-γ production by IL-12-responsive NK cells in RON−/− mice following endotoxin challenge

In mice, the IL-12-responsive IFN-γ-producing pool of cells includes NK, NKT, and innate T cells (6, 7). Innate T cells, defined as CD4+ or CD8+CD44hiCD122hi T cells, express markers of memory T cells but differ from them in being uniquely endowed with rapid effector function by virtue of elevated levels of transcription factors responsible for effector gene expression (29). To determine which group or groups of cells is responsible for the higher levels of IFN-γ in LPS-challenged RON−/− mice, we injected wild-type and RON−/− mice with 20 mg/kg LPS and then used flow cytometry to evaluate IFN-γ production by these different cell populations. Work done by Kambayashi et al. (6) has shown that the maximum in vivo production of IFN-γ by innate immune cells is at 3 h post-LPS injection. From 3 to 4 h following in vivo LPS challenge, we found that, by far, NK cells produced the most IFN-γ when compared with NKT and innate T cells (data not shown), and although in both un.injected and injected mice there were no differences in the percentage of splenic NK cells, we found that in RON−/− mice a higher percentage of NK cells produced IFN-γ (Fig. 2, A and B).

Although the greater percentage of IFN-γ-producing NK cells in the RON−/− mice can be explained by the higher levels of IL-12 detected in the serum and spleen of LPS-challenged RON−/− mice, it is also possible that the NK cells in RON-bearing mice are functionally less responsive due to the direct or indirect effects of RON signaling. It is well established that RON in expressed on tissue resident peritoneal macrophages and not on bone marrow-derived macrophages. It has, however, never been established whether or not RON is expressed on NK cells. To address this possibility, we sorted NK1.1+CD3− NK cells from the spleen of WT mice and compared the expression of RON on these cells to bone marrow-derived macrophages and peritoneal macrophages by real-time PCR. Our results show that NK cells express little or no RON compared with the prototypic RON-bearing cell type, the peritoneal macrophage (Fig. 2C). To compare the inherent responsiveness of NK cells from WT and RON−/− mice to cytokine stimulation, splenocytes from mice of both genotypes were stimulated with the same concentrations of IL-12, IL-18, and IL-2.
Table I. Increased serum levels of ASTa and ALT following d-GalN and LPS challenge in RON−/− mice

<table>
<thead>
<tr>
<th>20 mg of d-GalN + X µg of LPS</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RON+/+</td>
<td>RON−/−</td>
</tr>
<tr>
<td>0</td>
<td>261 ± 221 (n = 9)</td>
<td>159 ± 71 (n = 5)</td>
</tr>
<tr>
<td>0.01</td>
<td>228 ± 140 (n = 2)</td>
<td>238 ± 0 (n = 1)</td>
</tr>
<tr>
<td>0.1</td>
<td>295 ± 71 (n = 3)</td>
<td>217 ± 146 (n = 3)</td>
</tr>
<tr>
<td>1</td>
<td>290.8 ± 97 (n = 10)</td>
<td>603 ± 193** (n = 10)</td>
</tr>
<tr>
<td>10</td>
<td>634 ± 166 (n = 2)</td>
<td>2046 ± 6181 (n = 3)</td>
</tr>
<tr>
<td></td>
<td>58 ± 28 (n = 9)</td>
<td>45 ± 19 (n = 5)</td>
</tr>
<tr>
<td></td>
<td>81.5 ± 6 (n = 2)</td>
<td>51.0 ± 0 (n = 1)</td>
</tr>
<tr>
<td></td>
<td>172 ± 63 (n = 3)</td>
<td>110 ± 105 (n = 3)</td>
</tr>
<tr>
<td></td>
<td>197 ± 150 (n = 10)</td>
<td>504 ± 297** (n = 10)</td>
</tr>
<tr>
<td></td>
<td>657 ± 186 (n = 2)</td>
<td>2669 ± 451 (n = 3)</td>
</tr>
</tbody>
</table>

a, AST, aspartate aminotransferase; ALT, alanine aminotransferase.
b, WT and RON−/− CD-1 mice were injected i.p. with 20 mg of d-GalN in combination with the indicated doses of LPS. Serum levels of AST and ALT were assessed at 6 h postinjection.

Enhanced susceptibility of RON−/− mice to endotoxin challenge is mediated by IFN-γ

Previous studies from our laboratory have shown that RON−/− mice are more susceptible to LPS-induced sepsis syndrome (19). Another model of LPS-induced inflammatory disease is the LPS-d-GalN model. In this model, hepatocytes are sensitized to the effects of LPS-induced inflammatory cytokines by the liver-specific RNA synthesis inhibitor d-GalN (30). Inflammatory cytokines subsequently induced by LPS lead to fulminant hepatic failure characterized by massive hepatic damage evident by histology, and high levels of the liver enzymes aspartate aminotransferase and alanine aminotransferase in the serum, both markers of acute liver injury. To test the susceptibility of RON−/− mice to LPS-d-GalN-induced liver injury, WT and RON−/− CD-1 mice were injected with 20 mg of d-GalN and different doses of LPS. Although there were no clear differences in liver damage between WT and RON−/− mice at lower doses, there were clear and significant differences between the two strains at higher doses of LPS with the RON−/− mice showing the more adverse phenotype, as predicted (Table I).

Studies have shown that IFN-γR knockout mice are more resistant to in vivo LPS challenge with or without d-GalN (10). These studies have provided great insight into the role of IFN-γ in mediating LPS-induced inflammatory disease. To determine whether the increased susceptibility of RON−/− mice to in vivo LPS challenge is due to the higher levels of IFN-γ induced in these mice, we crossed RON−/− mice with IFN-γR−/− mice to generate DKO mice. Our previous studies with endotoxic shock were performed using mice on the CD-1 background (19). As the IFN-γR−/− mice are on the C57BL/6 background, we backcrossed the RON mutation 12 generations onto the C57BL/6 background. To confirm the increased susceptibility to in vivo endotoxin challenge on this background, WT and RON−/− mice were injected i.p. with 20 mg/kg LPS, and lethality was assessed over time. Although ~60% of the WT mice survived this dose of LPS, all of the RON−/− mice succumbed to septic shock. When challenged with 20 mg/kg LPS, all IFN-γR−/− and IFN-γR−/− × RON−/− DKO mice survived, reflecting the increased resistance of the IFN-γR−/− mice to in vivo endotoxin challenge (Fig. 3A). To determine whether the DKO mice showed increased susceptibility at higher doses, we challenged the IFN-γR−/− and DKO mice with 35 and 50 mg/kg LPS. Although we begin to see susceptibility of IFN-γR−/− mice to septic shock at these higher doses, there was no difference in susceptibility with or without RON (Fig. 3B). These data indicate that the increased susceptibility of RON−/− mice to shock is mediated by IFN-γ-dependent signals.

MSP activation of the RON receptor inhibits IFN-γ-mediated signaling in primary macrophages in vitro

The adverse phenotype of the RON−/− mice was overcome by crossing these mice with IFN-γR−/− mice. This suggested that the enhanced susceptibility of RON−/− mice to endotoxin challenge is due to signals emanating from the IFN-γ receptor. Although the higher levels of IFN-γ seen in LPS-challenged RON−/− mice could explain these findings, it is also possible that RON could be negatively regulating IFN-γ responses and that consequently the enhanced responsiveness of RON−/− macrophages to IFN-γ is contributing to this phenotype. Signaling through the RON receptor has been shown to down-regulate genes induced by a
combination of LPS and IFN-γ stimulation, such as IL-12p40 and iNOS (18, 21). The IL-12p40 and iNOS promoters contain both IFN-responsive and NFκB-binding elements. Although signaling through RON has been shown to down-regulate NF-κB transcripational activity in RON-expressing stable cell lines (31), the effect of MSP/RON signaling specifically on IFN-induced signals is yet to be elucidated. To independently assess the effects of MSP/RON signaling on IFN-γ-induced signals in macrophages, we stimulated peritoneal macrophages with MSP followed by a high dose of IFN-γ (200 U/ml) and assessed the surface expression of MHC class II, a classic IFN-γ-induced gene. Flow cytometric analysis showed that, whereas MSP stimulation alone did not significantly affect MHC class II expression on peritoneal macrophages (data not shown), MSP attenuated the induction of MHC class II by IFN-γ (Fig. 4A). This inhibition was not observed in macrophages from RON−/− mice, indicating that these effects of MSP are transmitted via the RON receptor (Fig. 4B).

The MHC class II gene regulator, CIITA (CIITA), plays an important role in the up-regulation of class II expression by IFN-γ. To determine whether RON signaling inhibits CIITA expression, we stimulated primary peritoneal macrophages with IFN-γ and assessed CIITA expression levels by RT-PCR. As expected, IFN-γ stimulation of these cells resulted in the enhanced expression of CIITA; however, MSP significantly inhibited this induction (Fig. 4C). This inhibition was not observed in peritoneal macrophages from RON−/− mice (Fig. 4D), indicating that this response is also mediated by the activation of the RON receptor.

IFN-γ stimulates expression of downstream target genes through a STAT1-dependent mechanism. Phosphorylation of the STAT1 Tyr701 residue is critical for its dimerization and nuclear translocation (32). We next examined the induction of STAT1 Tyr701 phosphorylation by IFN-γ in the presence and absence of MSP. Primary peritoneal macrophages were stimulated with or without MSP followed by IFN-γ. Subsequently, STAT1 tyrosine phosphorylation was assessed by Western blot analysis. The results shown in Fig. 5A demonstrate that STAT1 becomes phosphorylated following stimulation with IFN-γ and that this response is inhibited by MSP. To determine whether this signaling defect could be due to a MSP-induced decrease in IFN-γR expression, primary peritoneal macrophages were cultured in the presence or absence of MSP for 12 h, and the levels of IFN-γR expression were measured by flow cytometry. We observed a decrease in IFN-γR levels in the presence of MSP (Fig. 5B). Taken together, these results indicate that the MSP/RON signaling pathway negatively regulates the responsiveness of macrophages to IFN-γ-mediated signals at least in part at a receptor proximal level.

MSP induces up-regulation of SOCS1 and SOCS3 in primary peritoneal macrophages

SOCS1 has been identified as a critical negative regulator of IFN-γ signaling in vivo. Mice with a targeted deletion in SOCS1 die shortly after birth due to fatty degeneration and necrosis of the liver (33, 34). This phenotype is ameliorated when the mice are crossed with IFN-γ−/− animals, suggesting that the liver damage is due to enhanced susceptibility to IFN-γ (35). SOCS1 targets the phosphorylation of STAT1 via an SH2-dependent interaction with JAK2, by affecting the turnover of JAK2 and by restricting the access of STAT1 to JAK2 (36–38).

To determine whether the RON-mediated inhibition of IFN-γ-induced STAT1 tyrosine phosphorylation could be due to a MSP/RON-mediated induction of SOCS1, we used real-time PCR and examined the expression of SOCS1 in primary peritoneal macrophages following stimulation with MSP. As it has been shown that recombinant SOCS3 protects animals from the lethal effects of sepsis, we also assessed the induction of SOCS3 by MSP stimulation. IL-10, which has previously been shown to induce SOCS3 expression in primary macrophages (39), was used as a positive control in our study. Data from these studies indicate that MSP induces the expression of both SOCS1 and SOCS3 in primary peritoneal macrophages at 1 and 3 h after stimulation and that the expression of these genes returns to baseline by 6 h (Fig. 6). These data point to a mechanism by which RON could inhibit IFN-γ-induced signals and confer resistance to endotoxin challenge.
These results provide insight into the mechanism by which blots were performed using Abs to p-Stat3, Stat3, p-Stat1, and Stat1 (were cultured with or without 200 ng/ml MSP for up to 4.5 h. Western phosphorylation. Primary peritoneal macrophages from BALB/cJ mice were cultured with or without 200 ng/ml MSP for up to 4.5 h. SOCS3 promoter has shown that besides the two STAT3-binding activated STAT1 and SOCS3 by STAT3 (11). The analysis of the quantified by real-time PCR. Figure 6. MSP induces expression of SOCS1 and SOCS3 in primary peritoneal macrophages. Primary peritoneal macrophages from C57BL/6 mice were cultured in the presence of 100 ng/ml MSP for the indicated times, or IL-10 for 3 h. SOCS1 and SOCS3 gene expression levels were quantified by real-time PCR.

FIGURE 6. MSP induces expression of SOCS1 and SOCS3 in primary peritoneal macrophages. Primary peritoneal macrophages from BALB/cJ mice were cultured with or without 200 ng/ml MSP for up to 4.5 h. SOCS1 and SOCS3 gene expression levels were quantified by real-time PCR.

It is a well-studied fact that SOCS1 is strongly induced by activated STAT1 and SOCS3 by STAT3 (11). The analysis of the SOCS3 promoter has shown that besides the two STAT3-binding IFN-γ activation site elements, a proximal AP-1 binding site is critical for LPS-induced SOCS3 expression in macrophages and microglial cells (40). These studies also show that the inhibition of the ERK1/2 MAPK, compared with JNK and p38, results in the greatest inhibition of LPS-induced SOCS3 production. Furthermore, macrophages from c-Fos−/− mice are defective in SOCS1 and SOCS3 production in response to LPS stimulation or Salmonella infection (41). Taken together, these studies have shown the importance of STAT and AP-1 transcription factors in SOCS production. To determine whether signaling through RON can induce STAT1/3 and/or MAPK activation in primary peritoneal macrophages, we stimulated peritoneal macrophages with MSP and assessed STAT1 and 3, ERK1/2, JNK, and p38 phosphorylation by Western blot over time. Our data show that within 30 min the activation of the RON receptor by its ligand, MSP, results in the robust induction of ERK1/2 phosphorylation. In comparison, we see only a minor induction of p38 and JNK activation even after 4.5 h of MSP stimulation. Finally, we show that MSP induces significant STAT3 but not STAT1 phosphorylation in peritoneal macrophages within 1 h of receptor activation (Fig. 7). This induction was not observed in primary peritoneal macrophages from RON−/− mice (data not shown). These results provide an insight into the mechanism by which MSP/RON signaling could induce SOCS1 and SOC3 expression in macrophages.

**Discussion**

IL-12 is a key regulator of IFN-γ during endotoxemia, and NK cells stimulated by IL-12 are the largest producers of IFN-γ following LPS administration. Mice with targeted deletions of the period 2 (42) and receptor-interacting protein 2 (43) genes have been shown to be resistant to LPS-induced shock, due primarily to impaired IFN-γ production by NK cells, whereas CD137- and CD7-deficient mice are resistant to septic shock due to a reduction in NK/NKT cell populations (44, 45). Studies herein, demonstrating enhanced IL-12 expression in RON−/− mice following endotoxin challenge, before the onset of elevated systemic levels of IFN-γ, support a model in which RON regulates IFN-γ production by IL-12-responsive innate immune cells, by limiting LPS-induced IL-12p40 expression.

Whereas NK, NKT, CD4+, and CD8+ innate T cells all produced IFN-γ in our studies following LPS challenge (data not shown), NK cells appeared to be the predominant source of IFN-γ. Although there are no significant differences in the percentage of splenic NK cells before or after LPS challenge, there is a significant difference in the percentage of splenic NK cells producing IFN-γ following in vivo LPS challenge in RON−/− mice. The expression of the Tyro-3 family of receptor tyrosine kinases on NK cells affects the repertoire of activating and inhibiting receptors on and the overall functional maturity of, these cells (46). In contrast, our studies indicate that RON is not expressed on NK cells nor does it significantly affect the intrinsic ability of NK cells to produce IFN-γ in response to cytokine stimulation. Taken together, these data lend further credence to the idea that signaling through RON indirectly affects the production of IFN-γ by regulating IL-12 production.

IFN-γ stimulation of macrophages primes them for the enhanced production of a number of cytokines and cytotoxic molecules in response to TLR-generated signals. One of the underlying molecular mechanisms for this priming is mediated by the ability of IFN-γ-induced STAT1, and the subsequently generated IFN regulatory factor 1 and IFN consensus sequence-binding protein, to cooperate with TLR-induced NFκB to promote cytokine gene expression. Here we demonstrate that MSP/RON signaling in macrophages results in the down-regulation of IFN-γR expression and IFN-γ-induced STAT1 phosphorylation, CIITA up-regulation and MHC class II surface expression. These data clearly demonstrate that, in addition to regulating the production of IFN-γ, signaling through RON inhibits IFN-γ-mediated signals. It is possible that, in vivo, this inhibition could serve as part of a feedback mechanism to limit the action of IFN-γ and thus prevent excessive inflammation.

SOCS1 and SOCS3 are potent inhibitors of TLR and cytokine receptor signaling (11–13, 35). SOCS1 inhibits IFN-γ signaling via several mechanisms including: 1) binding to the activation loop of Jak2 and inhibiting the kinase activity of Jak2 through the killer Ig-related receptor domain which acts as pseudosubstrate; 2) ubiquitin-mediated degradation of Jak2 via the SOCS domain; and 3) binding to the IFN-γR, thus inhibiting the recruitment of STAT1 (36–38). We have shown that signaling through RON induces both SOCS1 and SOCS3 expression in primary macrophages. Although SOCS3 is typically not associated with the inhibition of IFN signaling, it inhibits signaling events common to the TLR-4 and IL-1 signaling pathways (47). Thus, SOCS3 could be responsible, in part, for the inhibition of LPS-induced IL-12p40 by MSP/RON, resulting in the elevated levels of IFN-γ observed in the RON−/− mice following LPS challenge. The induction of SOCS1 and 3

**FIGURE 7.** MSP RON signaling induces MAP kinase and STAT3 phosphorylation. Primary peritoneal macrophages from BALB/cJ mice were cultured with or without 200 ng/ml MSP for up to 4.5 h. Western blots were performed using Abs to p-Stat3, Stat3, p-Stat1, and Stat1 (A); and pErk, Erk, p-p38, p38, P-JNK, and JNK (B).
mRNA by MSP occurred within 1–3 h of stimulation. We have shown previously that the inhibition of macrophage activation by MSP requires 2–4 h of stimulation with MSP before activation of the cells with IFN-γ and LPS, consistent with a requirement for de novo protein synthesis (18).

SOCS1 is induced by IFN signaling as part of a classic negative feedback loop (11). Recently, it has been shown that the TAM receptor, Axl, can physically associate with the IFNAR1 to induce STAT1 in a ligand-dependent manner, which in turn induces SOCS1 and SOCS3 expression (14). However, our inability to detect significant STAT1 phosphorylation following stimulation suggests that the mechanism by which RON induces SOCS1 expression is distinct from that of the TAM receptors. IL-6 and IL-10 induce SOCS-3 via a STAT3-dependent mechanism (48). We have shown previously, using primary peritoneal macrophages from IL-10 knockout mice, that the inhibition of IL-12p40 by MSP is independent of IL-10. Furthermore, we have demonstrated that MSP does not induce IL-10 production by these cells, nor does it cooperate with LPS to produce enhanced levels of IL-10. Although we have shown that MSP can enhance LPS-induced IL-6, it does not induce IL-6 production in the absence of LPS (18). Therefore, we predict that RON induces the up-regulation of SOCS-3 expression via a parallel pathway involving STAT3.

We have shown previously that a N-terminally truncated form of murine RON (Sf-RON) induces the recruitment and phosphorylation of STAT3 through a docking-site tyrosine-dependent engagement of a GRB2/GAB2 signaling complex (49) and that this signaling pathway is required for the development of erythroleukemia initiated by the activation of Sf-RON. It is therefore possible that MSP/RON can mediate STAT3 phosphorylation in macrophages by directly signaling through a GRB2/GAB2 complex. In addition, whereas STAT proteins have become synonymous associated with SOCS expression, recent work has demonstrated that MAP kinases, especially ERK1/2, contribute significantly to the expression of SOCS1 and SOCS3 via the AP-1 transcription factor (41). Here we demonstrate that signaling through MSP/RON leads to the potent induction of ERK1/2 in primary peritoneal macrophages. It is therefore possible that the induction of ERK1/2 by RON also plays a pivotal role in its ability to induce SOCS-1 and SOCS-3.

In conclusion, our work here suggests that the RON receptor tyrosine kinase plays a key role in controlling the production of IFN-γ by NK cells in endotoxin-induced innate immune responses by regulating macrophage IL-12 production. Furthermore, our studies point to a model in which RON regulates the responsiveness of macrophages to IFN-γ by down-regulating IFN-γ induced tyrosine phosphorylation of STAT1 via the up-regulation of SOCS1. Finally, experiments with the RON and IFN-γR DKO mice suggest that the elevated levels of IFN-γ and enhanced responsiveness of macrophages to IFN-γ play a central role in the increased susceptibility of RON knockout mice to LPS challenge.

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Disclosures
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References