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CD11b/CD18 (Mac-1) Is a Novel Surface Receptor for Extracellular Double-Stranded RNA To Mediate Cellular Inflammatory Responses

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During viral infection, extracellular dsRNA is a potent signaling molecule that activates many innate immune cells, including macrophages. TLR3 is a well-known receptor for extracellular dsRNA, and internalization of extracellular dsRNA is required for endosomal TLR3 activation. Preserved inflammatory responses of TLR3-deficient macrophages to extracellular dsRNA strongly support a TLR3-independent mechanism in dsRNA-mediated immune responses. The present study demonstrated that CD11b/CD18 (Mac-1 [macrophage-1 Ag]), a surface integrin receptor, recognized extracellular dsRNA and induced macrophage immune responses. CD11b deficiency reduced inflammatory cytokine induction elicited by polyinosinic-polycytidylic acid (poly I:C; a synthetic dsRNA) in mouse sera and livers, as well as in cultured peritoneal macrophages. dsRNA-binding assay and confocal immunofluorescence showed that Mac-1, especially the CD11b subunit, interacted and colocalized with poly I:C on the surface of macrophages. Further mechanistic studies revealed two distinct signaling events following dsRNA recognition by Mac-1. First, Mac-1 facilitated poly I:C internalization through the activation of PI3K signaling and enhanced TLR3-dependent activation of IRF3 in macrophages. Second, poly I:C induced activation of phagocyte NADPH oxidase in a TLR3-independent, but Mac-1-dependent, manner. Subsequently, phagocyte NADPH oxidase-derived intracellular reactive oxygen species activated MAPK and NF-κB pathways. Our results indicate that extracellular dsRNA activates Mac-1 to enhance TLR3-dependent signaling and to trigger TLR3-independent, but Mac-1-dependent, inflammatory oxidative signaling, identifying a novel mechanistic basis for macrophages to recognize extracellular dsRNA to regulate innate immune responses. This study identifies Mac-1 as a novel surface receptor for extracellular dsRNA and implicates it as a potential therapeutic target for virus-related inflammatory diseases. The Journal of Immunology, 2013, 190: 115–125.

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As the first line of host defense, the innate immune system combats numerous pathogens through a diverse set of pattern recognition receptors (PRRs). These PRRs are capable of identifying pathogen-associated molecular patterns (PAMPs) and initiating the innate immune response. During viral infections, various virus-associated PAMPs are recognized by, and bind to, their respective PRRs, which induces robust host antiviral and immune responses. It is well known that viral-produced dsRNA is the first line of host defense, the innate immune system combats numerous pathogens through a diverse set of pattern recognition receptors (PRRs). These PRRs are capable of identifying pathogen-associated molecular patterns (PAMPs) and initiating the innate immune response. During viral infections, various virus-associated PAMPs are recognized by, and bind to, their respective PRRs, which induces robust host antiviral and immune responses. It is well known that viral-produced dsRNA
and damage-associated molecular patterns, such as Gram-negative bacteria–derived LPS (13), aggregated β-amyloid (14), and damage-associated alarmin HMGB1 (15). Mac-1, expressed on many innate immune cells, such as monocytes, granulocytes, macrophages, and NK cells (16), has been implicated in various immune cell responses, including adhesion, migration, phagocytosis, chemotaxis, cellular activation, and cytotoxicity (17, 18). Furthermore, Mac-1 was reported to participate in inflammatory diseases associated with Ross River virus infection (19) and to bind some nucleotides, such as oligodeoxynucleotid (20). These characteristics of Mac-1 prompted us to investigate the possibility that it serves as a PRR for extracellular dsRNA to regulate the innate immune response.

In this study, we identified Mac-1 as a novel surface receptor mediating extracellular dsRNA-elicted cellular immune responses. Our results demonstrate that Mac-1 can recognize extracellular dsRNA on the cell surface and then mediate outside-in signaling, regulate dsRNA internalization, and mediate activation of phagocyte NADPH oxidase (NOX2) to induce cellular immune responses in macrophages. Our results provide new insight into how the macrophage recognizes extracellular signals associated with lytic virus infections and mediates the innate immune response.

Materials and Methods

Animal study

CD11b−/− mice (Mac-1–deficient), gp91−/− mice (NADPH oxidase–deficient), and their age-matched wild-type (WT) control (CD11b+/+ and gp91+/+) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR3−/− mice and their age-matched WT control (TLR3+/+) were also obtained from The Jackson Laboratory. Housing and breeding of the animals were performed humanely and with regard for alleviation of suffering following the National Institutes of Health’s Laboratory Animals (Institute of Laboratory Animals 1996). Six–to-eight-week-old male mice of different strains were used in all experiments. All procedures were approved by the National Institutes of Environmental Health Sciences Animal Care and Use Committee.

An in vivo animal model involved immune activation by poly I:C (Sigma-Aldrich, St. Louis, MO; an average size of 300–750 bp). WT mice and CD11b−/− (CD11b-knockout [KO]) mice were injected i.p. with poly I:C (5 mg/kg). Serum was collected 2 h later for cytokine measurement using commercially available ELISA kits (R&D Systems, Minneapolis, MN), and liver tissues were harvested for mRNA isolation and cytokine assay by quantitative real-time PCR.

Preparation of peritoneal macrophages and culture of macrophage cell line

Peritoneal macrophages from different strains were induced and harvested, as previously described (21). Briefly, WT mice, CD11b-KO mice, and gp91−/− (gp91-KO) mice were injected i.p. with 2 ml 3% thioglycollate. After 4 d, peritoneal exudate macrophages were collected by lavage in 5 ml ice-cold RPMI 1640 medium (Invitrogen, CA), washed twice in RPMI 1640, and preincubated in serum-free medium for 1 h. The cells were then washed twice to remove nonadherent cells. Adherent macrophages were cultured in RPMI 1640 medium containing 10% FBS (Invitrogen), 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2.

The murine macrophage cell line RAW 264.7 (ATCC TIB-71) was suspension cultured in DMEM containing 10% FBS, 2 mM-L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2.

dsRNA-binding assay

The dsRNA-binding assay was performed as described previously (22, 23). Briefly, poly I:C–conjugated beads, poly I:C–conjugated beads, or noncoated empty beads were equilibrated in lysis buffer containing RNase inhibitor (Invitrogen) and incubated overnight with whole-cell lysates at 4°C on a rotator. After centrifugation, beads were washed extensively and then resuspended in sample buffer (Invitrogen). Samples were boiled for 10 min and centrifuged at 13,000 × g for 1 min to discard insoluble pellets. Samples were then loaded onto SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes, and probed by immunoblot analysis against CD11b (Abcam, MA).

Flow cytometric analysis

Poly I:C was labeled with FITC using a Mirus RNA labeling kit (24). Labeled RNA was then purified with an RNeasy mini kit (Qiagen, CA). Peritoneal macrophages or RAW 264.7 cells were washed twice and suspended in HBSS (Invitrogen). The cells (1 × 10⁶ cells/ml) were kept on ice for 10 min to determine the surface binding of poly I:C by incubating with 10 µg/ml FITC-labeled poly I:C for 20 min (20). After washing three times with cold HBSS, poly I:C–bound macrophages were detected by a BD LSR II Flow Cytometer. A live cell gate was made using forward versus side scatter plot. The surface binding was expressed as the mean fluorescence intensities of the total calculated population. Internalization analysis of FITC-labeled poly I:C was performed using the same procedure as described above for the surface binding process, with the exception that cells were incubated in 37°C instead of on ice (20), and trypan blue (1 mg/ml) was used to quench extracellular surface-bound fluorescence, as described previously (25). The internalization of poly I:C was expressed as the mean fluorescence intensities of the total calculated population.

Confocal microscopy

Poly I:C and poly C were labeled with Cy3 for confocal observation (24). Peritoneal macrophages were seeded and grown in glass-bottom microwell dishes (MatTek, MA). Cells were then treated with 10 µg/ml Cy3-labeled poly I:C or poly C, either on ice for 20 min to observe the surface binding or at 37°C for 15 or 30 min to determine the internalization. Stained macrophages were washed three times with ice-cold PBS and fixed with 4% paraformaldehyde for further observation on a Zeiss LSM510 Laser Scanning Confocal Microscope (Carl Zeiss MicroImaging, Germany).

Quantitative real-time PCR

Total RNA of cells or tissues was isolated using TRIzol reagent and then first-strand cDNA was synthesized using SuperScript Reverse Transcriptase (Invitrogen), according to the manufacturer’s protocols. After 0.5 µg total RNA was subjected to a reverse-transcription reaction, 2 µl cDNA was amplified by quantitative real-time PCR analysis for the induction of inflammatory cytokines using SYBR Green Master mix (Bio-Rad, CA) in a final volume of 25 µl. The following primers were used: mouse TNF-α: 5′-CATCCTTCTCAAATTTGAGTGGCACA-3′ (forward), 5′-TGGGAG-TAGAAGAGTGATAC-AACC-3′ (reverse); mouse IL-12p40: 5′-GGAG-GACCCGAGACGATATA-3′ (forward), 5′-AACCTGGAAGGGAAGTGG-AATGG-3′ (reverse); mouse IFN-β: 5′-ATGATGTTGTTGCAGC-3′ (forward), 5′-TGACCTTAAAACTGAGTAACTGTA-3′ (reverse); mouse IL-6: 5′-TGCGCTTCTTGGAAGTATGC-3′ (forward), 5′-GTATCCTC-TCTGAAGAAGCTG-3′ (reverse); mouse GAPDH: 5′-TTTCCACCATGGAGAAGGC-3′ (forward), 5′-GGCATGGACTGGTGTAC-3′ (reverse). Data were normalized to GAPDH expression.

Measurement of extracellular superoxide and intracellular reactive oxygen species

The release of superoxide was determined by measuring the fluorescence dismutate (SOD)–inhibitable reduction of tetrazolium salt (2-[4-iodo-phenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium [WST-1] as described (26). Briefly, peritoneal macrophages (1 × 10⁶/well) were grown overnight in 96-well plates in DMEM medium containing 10% FBS and switched to phenol red-free HBSS (100 µl/well). Fifty microliters of HBSS containing vehicle, poly I:C, or PMA was then added to each well, followed by the addition of 10 µl WST-1 (1 mM in HBSS, with or without 600 U/ml SOD). The cells were incubated for 30 min at 37°C, and the absorbance was read at 450 nm with a SpectraMax Plus microplate spectrophotometer (Molecular Devices, CA).

The production of intracellular reactive oxygen species (iROS) was measured by the fluorescence probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA), as described (26). Briefly, macrophages cultured overnight in 96-well plates were incubated with 10 µM DCFH-DA (Invitrogen) for 30 min at 37°C. After two washes with HBSS buffer, cells were switched to HBSS containing 1% FBS. After the cells were incubated with vehicle or poly I:C (50 µg/ml) at 37°C for 30 min, the fluorescence density was read.
at 488 nm for excitation and at 525 nm for emission using a SpectraMax Gemini XS fluorescence microplate reader (Molecular Devices).

**Nuclear extraction, gel electrophoresis, and Western blotting analysis**

Peritoneal macrophages from WT and CD11b−/− mice were incubated with poly I:C (50 μg/ml) or vehicle at 37°C for 60 or 120 min and washed twice with cold PBS. Nuclear extraction was performed at 4°C with a nuclear extraction kit (Affymetrix, following the manufacturer’s instructions). Protein concentrations were determined using the DC protein assay (Bio-Rad). The whole-cell lysates from cultured cells were homogenized in radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1:100 protease inhibitor mixture) with phosphatase inhibitors (10 mM NaF, 1 mM Na4P2O7, and 1 mM sodium orthovanadate), sonicated, and boiled for 10 min. Protein concentrations were determined using the bicinchoninic acid assay (Pierce). Protein samples were resolved on 4–12% SDS-PAGE, and immunoblot analysis was performed using Abs against indicated signaling molecules (Cell Signaling Technology). An Ab against GAPDH (Sigma-Aldrich) or HDAC2 (Santa Cruz Biotech) was included as an internal standard to monitor loading errors.

**Immunofluorescence staining**

Macrophages were grown and treated on glass coverslips, fixed in 4% paraformaldehyde, permeabilized with 0.4% Triton X-100, and blocked with 5% goat serum. Samples were incubated sequentially with primary Abs and Alexa Fluor-conjugated secondary Abs (Invitrogen) and then analyzed using a Zeiss LSM510 Confocal Microscope. To study the distribution and colocalization of desired proteins, we analyzed images from ≥10 random fields from three independent experiments.

**Statistical analysis**

Data are expressed as mean ± SEM. Statistical significance was assessed by ANOVA, followed by the Bonferroni t-test, using GraphPad Prism software (GraphPad Software, CA). A p value < 0.05 was considered statistically significant.

**Results**

**Mac-1−deficient mice exhibited impaired immune response to poly I:C**

To determine whether Mac-1 is a potential candidate in sensing extracellular dsRNA and regulating the immune response, mice deficient in the CD11b subunit of Mac-1 and age-matched WT mice were injected i.p. with synthetic dsRNA poly I:C (5 mg/kg).

Measurement of serum inflammatory factors 2 h after poly I:C injection revealed decreased circulating TNF-α, IFN-β, and IL-12p40 in CD11b−/− mice compared with WT controls (Fig. 1A). Similarly, the mRNA level of TNF-α, IL-12p40, IFN-β, and IL-6 in the liver of CD11b−/− mice was reduced (Fig. 1B). Collectively, Mac-1−deficient (CD11b−/−) mice displayed an impaired immune response to poly I:C.

**Mac-1 deficiency impaired immune responses to poly I:C in macrophages**

Because Mac-1 is expressed dominantly on macrophages that have a central role in the innate immune response, we next compared inflammatory cytokine induction and investigated inflammatory signaling cascades after poly I:C stimulation in CD11b−/− and WT peritoneal macrophages. Consistent with our in vivo results, the mRNA level of TNF-α, IL-12p40, IFN-β, and IL-6 was significantly lower in CD11b−/− macrophages than in WT controls (Fig. 2A). Secreted TNF-α and IL-12p40 in the supernatant showed a significant reduction in CD11b−/− macrophages (Fig. 2B). IL-12p70 was detected after macrophages were challenged with 50 μg/ml poly I:C for 24 h, reaching 2.8 ± 0.3 pg/ml in WT macrophages and 0.8 ± 0.6 pg/ml in CD11b−/− macrophages. A similar pattern was observed for the poly I:C–elicited release of NO, an inflammatory molecule (Fig. 2C). In contrast, stimulation of cultured peritoneal macrophages with TNF-α (50 ng/ml) or PMA (100 nM) induced indistinguishable immune responses in CD11b−/− macrophages and WT macrophages (Supplemental Fig. 1). In addition, TLR3 expression in CD11b−/− and WT macrophages was not different (Supplemental Fig. 2). Together, these data suggest that the observed impairment in the immune response of CD11b−/− macrophages to poly I:C is due to the functional deficiency of Mac-1 receptor to poly I:C stimulation and is not due to a general immune deficit in these cells.

**Poly I:C bound to Mac-1 receptor on the cell surface of macrophages**

A dsRNA-binding assay using cell extracts from peritoneal macrophages or RAW 264.7 cells (a mouse macrophage–like cell line) was used to test the possibility that poly I:C binds to Mac-1.

**FIGURE 1.** Mac-1−deficient mice exhibited impaired immune response after poly I:C injection. WT or CD11b−/− mice were injected i.p. with 5 mg/kg poly I:C. After 2 h, blood and livers were collected for analysis of inflammatory cytokine induction. The amount of TNF-α, IL-12p40, and IFN-β in serum was detected by ELISA (A), and the mRNA level of TNF-α, IFN-β, IL-6, and IL-12p40 in livers was measured by RT-PCR (B). Data are mean ± SEM from five to seven pairs of WT and CD11b−/− mice. *p < 0.01 versus vehicle-injected WT mice, †p < 0.01 versus poly I:C–injected WT mice.
Poly I:C–conjugated agarose beads pulled down the CD11b subunit of Mac-1, whereas unconjugated beads or beads conjugated with single-stranded poly C failed to do so (Fig. 3A). These results indicate that Mac-1 can specifically recognize double-stranded poly I:C. To further confirm the interaction between poly I:C and Mac-1, a surface binding assay was performed using flow cytometry and confocal imaging. FITC-labeled poly I:C bound to the surface of WT macrophages, whereas such surface binding was dramatically decreased in CD11b−/− macrophages (Fig. 3B, 3C). Furthermore, the surface binding was also significantly reduced in WT macrophages or RAW 264.7 cells pretreated with fibrinogen, an endogenous ligand of Mac-1, whereas such an inhibitory effect of fibrinogen was not observed in CD11b−/− macrophages (Fig. 3B, 3C). Consistent with this result, confocal imaging (Fig. 3D) showed that CD11b deletion or fibrinogen pretreatment reduced the surface binding of poly I:C in macrophages. In addition, immunofluorescence staining of CD11b revealed colocalization of CD11b with surface-bound Cy3–poly I:C in WT macrophages (Fig. 3E). Further overlapping analysis showed >75% overlap of Mac-1 with Cy3–poly I:C on the cell surface (Fig. 3E). Overall, these data provide strong evidence that Mac-1 can recognize and bind double-stranded poly I:C on the surface of the macrophage, indicating that Mac-1 may mediate immune responses to poly I:C through outside-in signaling.

**Mac-1 facilitated the internalization of poly I:C**

It is well known that internalization of extracellular dsRNA is a crucial step to subsequent antiviral responses induced by endosomal TLR3 activation (6, 11, 24). Our confocal imaging analysis detected intracellular poly I:C within 15 min after Cy3-labeled poly I:C was added to macrophages, and further accumulation of intracellular poly I:C was observed with extended time (30 min). However, the uptake of poly I:C was significantly attenuated in CD11b−/− macrophages compared with WT macrophages (Fig. 4A). In contrast to the differential internalization of poly I:C, the uptake of Cy3-labeled poly C (ssRNA) was indistinguishable in WT and CD11b−/− peritoneal macrophages, indicating the specificity of CD11b on dsRNA poly I:C (Fig. 4A). The flow cytometric analysis revealed ~40% reduction in the uptake of FITC-labeled poly I:C into CD11b−/− macrophages compared with WT macrophages (Fig. 4B). These results indicate a critical role for Mac-1 in the internalization of poly I:C into macrophages. Such a conclusion was confirmed by the finding that Mac-1–blocking Ab significantly attenuated the uptake of poly I:C into WT macrophages but not CD11b−/− macrophages (Fig. 4C).
As a common cellular signaling pathway of integrins (27, 28), the activation of PI3K was specifically described in previous studies on Mac-1–signaling transduction (29, 30). PI3K was also implicated in the regulation of endocytosis and intracellular membrane trafficking in macrophages (31, 32). In this study, we found that wortmannin, a PI3K inhibitor, blocked the uptake of poly I:C into the macrophage (Fig. 4D). Importantly, when challenged with poly I:C, the phosphorylation of AKT, a key kinase downstream of PI3K, was significantly impaired in CD11b\(^{−/−}\) macrophages (Fig. 4E). Taken together, the reduced uptake of poly I:C in the setting of PI3K inhibition in WT macrophages and the diminished PI3K activation in CD11b\(^{−/−}\) macrophages suggest that Mac-1 promotes the internalization of poly I:C through activating the PI3K pathway. Thus, Mac-1 may facilitate immune responses in macrophages through enhancing the uptake of extracellular poly I:C into the endosome, where TLR3 resides.

**Mac-1 altered poly I:C–induced downstream signaling**

It is well known that poly I:C activates IRF3 signaling via TLR3, leading to the induction of IFN and IFN-inducible genes. The attenuation of poly I:C–elicited IFN-β induction in CD11b\(^{−/−}\) macrophages and mice (Fig. 1) suggests that Mac-1 might par-

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**FIGURE 3.** Poly I:C bound to Mac-1 receptor on the cell surface. dsRNA-binding assay was performed using poly I:C–coated agarose beads, un-conjugated beads, or poly C–coated beads. (A) Only poly I:C–coated beads can pull down the CD11b subunit of Mac-1 in either primary macrophages or RAW264.7 macrophage cells. (B-E) Surface-binding assay further delineated the interaction between poly I:C and Mac-1 on the cell surface. (B and C) Flow cytometry showed that WT macrophages had higher surface binding of FITC-labeled poly I:C than did CD11b\(^{−/−}\) macrophages, and the poly I:C binding was significantly inhibited by fibrinogen (1 μM) in WT macrophages and RAW264.7 cells but not in CD11b\(^{−/−}\) macrophages. (D) The confocal experiments revealed that more Cy3-labeled poly I:C bound to the surface of WT macrophages than to the surface of CD11b\(^{−/−}\) macrophages, and the binding of Cy3-labeled poly I:C was inhibited by fibrinogen only in WT macrophages. (E) CD11b immunostaining showed that Mac-1 colocalized with surface-bound Cy3–poly I:C. Data are mean ± SEM from three independent experiments in triplicate. *p < 0.01 versus poly I:C–treated groups, #p < 0.01 versus poly I:C–treated WT groups.
Data are mean ± SEM from three independent experiments in triplicate. *p < 0.01 versus vehicle-treated controls, #p < 0.01 versus poly I:C–treated WT groups.

FIGURE 4. Mac-1 facilitates the internalization of poly I:C. Cultured peritoneal macrophages from WT and CD11b<sup>−/−</sup> mice were incubated with Cy3-labeled poly I:C (10 μg/ml) at 37°C for 15 or 30 min or Cy3-labeled poly C (10 μg/ml) for 30 min. (A) Confocal imaging shows decreased intracellular Cy3 fluorescence in CD11b<sup>−/−</sup> macrophages compared with WT macrophages after these cells were treated with Cy3-labeled poly I:C but not with Cy3-labeled poly C. (B) Macrophages from WT and CD11b<sup>−/−</sup> mice were incubated with FITC-labeled poly I:C (10 μg/ml) at 37°C for 30 min. After the fluorescence of extracellular surface-bound FITC-labeled poly I:C was quenched by trypan blue (1 mg/ml), the fluorescence density of the internalized FITC-labeled poly I:C was measured by flow cytometry and was present after the subtraction of nonspecific autofluorescence in untreated control cells. (C) After pretreatment with normal IgG (2.5 μg/ml) or anti-CD11b Ab (2.5 μg/ml) for 15 min, macrophages from WT and CD11b<sup>−/−</sup> mice were incubated with Cy3-labeled poly I:C for 30 min. Confocal imaging showed reduced fluorescence density in the Ab-treated group. (D) The pretreatment of WT macrophages for 15 min with PI3K inhibitor wortmannin attenuated the uptake of Cy3-labeled poly I:C. (E) Western blot analysis on poly I:C–challenged WT and CD11b<sup>−/−</sup> macrophages at different time points. Impaired AKT phosphorylation was observed in CD11b<sup>−/−</sup> macrophages. Data are mean ± SEM from three independent experiments in triplicate. *p < 0.01 versus vehicle-treated controls, #p < 0.01 versus poly I:C–treated WT groups.

To further determine the downstream-signaling cascades of poly I:C–elicited Mac-1 activation, the MAPK and NF-κB pathways, two major signaling pathways responsible for proinflammatory cytokine induction, were assayed in WT and CD11b<sup>−/−</sup> macrophages. WT macrophages exhibited time-dependent phosphorylation in the MAPK and NF-κB pathways (Fig. 5A, 5C) after poly I:C (50 μg/ml) stimulation. CD11b<sup>−/−</sup> macrophages displayed a significant reduction in the phosphorylation of JNK1/2, the p65 subunit of NF-κB, and 1κB, as well as the degradation of 1κB-α (Fig. 5B, 5C). The phosphorylation of p38 was also impaired in CD11b<sup>−/−</sup> macrophages, although its reduction was less prominent than the reduction in JNK phosphorylation (Fig. 5B). These results indicate an important role for Mac-1 in the activation of JNK and NF-κB by poly I:C. We next treated WT macrophages with either a JNK inhibitor (SP600125; 5 μM) or an NF-κB inhibitor (Compound A; 1 μM) (33) for 30 min and then challenged the cells with poly I:C. Both inhibitors significantly suppressed the release of TNF-α and IL-12p40, which further demonstrated the importance of the activation of JNK and NF-κB in the poly I:C–induced immune response (Fig. 5D). Together, these results suggest that Mac-1 contributes to the poly I:C–induced immune response in macrophages through promoting the activation of the MAPK and NF-κB pathways.

In response to extracellular dsRNA, NF-κB activation is thought to be involved in the downstream signaling of TLR3 activation (7), but the engagement of TLR3 does not seem to be required for the activation of MAPK signaling in macrophages (34). Thus, our findings described above (Fig. 5) suggest that, in addition to the contribution to the TLR3–NF-κB pathway by facilitating dsRNA internalization, Mac-1 may induce TLR3-independent signaling pathways, such as the MAPK pathway. Bafilomycin A (BFA), an inhibitor of vacuolar-type H<sup>+</sup>-ATPase, blocks the acidification of the endosome and, therefore, inhibits TLR3 activation (35). In the presence of BFA, considerable induction of the proinflammatory cytokines TNF-α and IL-6 persisted after poly I:C treatment, whereas the induction of IFN-β was abolished (Supplemental Fig. 3). These data support the premise that type I IFN induction by extracellular poly I:C is TLR3 dependent (36, 37), but they also suggest that other proinflammatory cytokines can be induced by extracellular poly I:C in a TLR3-independent manner. Interestingly, the persisting levels of TNF-α and IL-6 in the setting of BFA were significantly lower in CD11b<sup>−/−</sup> macrophages than in WT controls, further supporting the involvement of Mac-1 in a TLR3-independent response to extracellular poly I:C.
Mac-1–dependent, TLR3-independent activation of NOX2 enhanced immune responses to poly I:C

Previous studies showed that Mac-1 ligands can induce the production of superoxide free radicals in macrophages and neutrophils where NOX2 acts as a major source of superoxide during inflammation (20, 30). Therefore, we examined the effect of the interaction of poly I:C and Mac-1 on NOX2 activation in macrophages. As shown in Fig. 6A, poly I:C (50 μg/ml) induced significant production of extracellular superoxide in WT macrophages. On the contrary, poly I:C failed to do so in macrophages deficient in CD11b or gp91 (the catalytic subunit of NOX2). However, PMA (a commonly used NOX2 stimulator) triggered robust superoxide production in both WT and CD11b−/− macrophages; as expected, PMA was inactive in gp91−/− macrophages. Although superoxide radical is membrane impermeable, its downstream products, H2O2 and peroxynitrite (the reaction product of superoxide and NO), are membrane permeable. Consistent with extracellular superoxide production, iROS were also elevated after poly I:C treatment in WT macrophages, whereas deficiency in either Mac-1 or NOX2 abolished such iROS production (Fig. 6B). These results indicate that poly I:C activates NOX2 to secrete superoxide anion in a Mac-1–dependent manner.

Next, we demonstrated the contribution of NOX2 activation to poly I:C-elicited induction of proinflammatory cytokines. The production of TNF-α and IL-12p40 in poly I:C-treated macrophages was significantly attenuated by the genetic deletion of gp91 (Fig. 6C) or by the pharmacological inhibition of NOX2 activity by apocynin, a widely used NOX2 inhibitor (Fig. 6D). The downstream signaling of NOX2 activation was determined in WT and gp91−/− macrophages. The phosphorylation of p38, JNK, and p65 was attenuated in gp91−/− macrophages compared with WT macrophages (Fig. 6E). Such reduced phosphorylation of p38, JNK, and p65 in gp91−/− macrophages was also observed in Mac-1–deficient macrophages (Fig. 5B, 5C). These results, combined with the finding that Mac-1 is required for poly I:C–induced NOX2 activation (Fig. 6A, 6B), indicated that poly I:C-elicited activation of Mac-1 activated NOX2 and, thereby, stimulated the MAPK and NF-κB pathways to promote proinflammatory cytokine production.

Interestingly, poly I:C–induced superoxide production was not altered in TLR3−/− macrophages compared with WT cells (Fig. 6F). In addition, the inhibition of NOX2 activity by apocynin led to a similar reduction in the induction of TNF-α and IL-12p40 in TLR3−/− and WT peritoneal macrophages (Fig. 6G). These results indicate that TLR3 was not involved in the activation of
NOX2. Altogether, our findings demonstrated that poly I:C activates NOX2 to release superoxide anion and to participate in the induction of proinflammatory cytokines in a TLR3-independent, but Mac-1–dependent, manner (Fig. 7). In addition, through facilitating the internalization of poly I:C, Mac-1 activation may amplify TLR3-dependent proinflammatory responses to dsRNA (Fig. 7).

Discussion

Extracellular dsRNA, as a potent stimulator of the innate immune response, induces inflammatory cytokines and chemokines in many cell types. However, the precise mechanism underlying its extracellular recognition and intracellular signaling remains largely unknown. In this study, we demonstrate that Mac-1 functions as a novel surface receptor for dsRNA in macrophages, as summarized in Fig. 7. Specifically, Mac-1 binds extracellular dsRNA on the surface of macrophages to mediate cellular immune responses. Further mechanistic studies indicate that Mac-1 activation facilitates the internalization of dsRNA and also activates NOX2, which enhances TLR3-dependent proinflammatory responses and triggers TLR3-independent oxidative immune responses. Thus, Mac-1 plays a distinct role in dsRNA-induced immune responses. Our findings suggest that Mac-1 acts as a bona fide PRR for extracellular dsRNA to signal downstream inflammatory responses.

As a member of β2 integrins, Mac-1 has long been recognized to participate in immune responses to infection and was suggested to be a therapeutic target (18, 38). For instance, Mac-1 (also known as CR3) can bind complement components (such as iC3b) induced by invading pathogens to activate innate and adaptive immune responses (39, 40). CD11b-deficient mice were used to uncover the specific contribution of Mac-1 to many immune responses, such as FcR-triggered inflammation (41), phagocytosis of complement-opsonized particles (42), and immune reactions to bacteria or LPS (13, 21). Recent studies of Ross River virus (RRV)-associated arthritis/myositis indicated a role for Mac-1 in virus-induced inflammation (19). RRV causes severe leukocyte-mediated inflammatory responses in joint and skeletal muscle tissues, leading to a chronic inflammatory manifestation similar to arthritis/myositis. CD11b<sup>−/−</sup> mice exhibit decreased proinflammatory and cytotoxic effectors (e.g., S100A9/S100A8 and IL-6) and less severe tissue damage compared with WT mice, indicating the contribution of Mac-1 to RRV-induced chronic inflammation. Although the investigators considered the activated complement components to be the stimulus for Mac-1, a direct
The role of Mac-1 in dsRNA-mediated signaling in the macrophage. The membrane receptor TLR3 recognizes and binds internalized extracellular dsRNA only in acidified endosomes and then activated TLR3 induces type I IFN production via IRF3 and proinflammatory cytokine generation via the NF-κB pathway. The present study identifies Mac-1 (CD11b/CD18 or CR3) as a novel PRR on the surface of macrophages. Mac-1 senses and binds extracellular dsRNA to facilitate the endocytosis of extracellular dsRNA, thereby amplifying the TLR3-dependent signaling, or to activate oxidative enzyme NOX2 to produce ROS, thereby activating the MAPK and NF-κB pathways to induce proinflammatory cytokine production in a TLR3-independent manner.

link between complement and Mac-1 was not demonstrated (19). Viral-produced dsRNA is a powerful viral PAMP, and it stimulates both innate and adaptive antiviral immune responses (1). Previous studies showed that Mac-1 acts as a membrane-bound PRR and binds a number of PAMPs and damage-associated molecular patterns (22, 42, 43). Further studies to define the role of viral dsRNA and Mac-1 activation in virus infection and host antiviral inflammatory response will identify novel disease mechanisms related to virus pathogens.

CD11b<sup>−/−</sup> mice displayed impaired immune responses to poly I:C, suggesting that Mac-1 may contribute to viral dsRNA-induced inflammation (Fig. 1). A dsRNA-binding assay and a cell surface–binding assay revealed that Mac-1 can recognize poly I:C on the surface of macrophages, indicating that it is a novel surface PRR for dsRNA (Fig. 3). The ligand-binding ability of Mac-1 may be due to intrinsic properties of the I-domain (the major binding site for Mac-1 ligands) in the CD11b subunit (44). The I-domain is known to interact with a large array of ligands. However, the binding profile of the I-domain is still unclear. The blockade of poly I:C binding by the I-domain–binding ligand fibrinogen (Fig. 3B–D) and the attenuation in poly I:C internalization by the anti-CD11b Ab specific for residues 250–350 within the I-domain (Fig. 4C) suggest that poly I:C may bind to the I-domain of CD11b. Structural analysis of the I-domain reveals a metal ion–dependent adhesion site (44); this site is thought to prefer negative-charged groups, such as aspartate or glutamate residues of ligands (e.g., ICAM-1) (18). Such coordination may also be important for the binding of the negative-charged phosphate group of poly I:C to this domain. However, our dsRNA-binding assay indicated that the recognition was specific for dsRNA (poly I:C), but not for ssRNA (poly C), which showed that the negative-charged group is not a determining factor for the binding of poly I:C to Mac-1.

We next demonstrated that Mac-1 promotes endocytosis of extracellular poly I:C. In fact, several reports revealed the involvement of Mac-1 in the phagocytosis of its ligands (20, 42), but little is known about the underlying mechanism. For dsRNA entry, type A scavenger receptors (SR-As) can mediate its internalization in fibroblasts (6) or epithelial cells (24). Consistent with these findings, inhibitors of SR-As (fucoidan or dextran sulfate) blocked the uptake of poly I:C into macrophages (Supplemental Fig. 4). The reduction in poly I:C internalization in macrophages deficient in CD11b or pretreated with an anti-CD11b Ab (Fig. 4A–C) indicates the participation of Mac-1 in the endocytosis of poly I:C. Thus, Mac-1 may assist or regulate the function of SR-As in the uptake of dsRNA. A recent study reported that Mac-1 and SR-As mediated the phagocytosis of degenerated myelin by macrophages through a PI3K-dependent pathway (45). Blockage of poly I:C entry into macrophages by a PI3K inhibitor (Fig. 4D) and attenuation of PI3K activation in CD11b<sup>−/−</sup> macrophages (Fig. 4E) imply that Mac-1 facilitates the endocytosis of poly I:C through activation of the PI3K pathway. The endocytosis of extracellular dsRNA is a critical step for antiviral TLR3 activation to induce type I IFN production via IRF3 (36, 37, 46). Although TLR3 partially localizes to the surface in some cell types (47–49), it only binds dsRNA in the low-pH endosome (11). The reduction in poly I:C internalization (Fig. 4A, 4B), IRF3 activation (Fig. 5A), and production of type I IFN (IFN-β) in CD11b<sup>−/−</sup> macrophages (Fig. 2A) indicates that Mac-1–mediated endocytosis of poly I:C participates in the TLR3-dependent antiviral response.

TLR3 is a well-established receptor for extracellular dsRNA. However, extracellular dsRNA is still able to induce a significant amount of multiple proinflammatory cytokines in TLR3<sup>−/−</sup> macrophages or microglia (7, 12). The TLR3-independent inflammatory responses triggered by dsRNA were confirmed by our similar findings (Fig. 6G). Most importantly, the current study delineated a TLR3-independent, but Mac-1–dependent, mechanism that underlines NOX2–associated oxidative immune responses to extracellular dsRNA. First, poly I:C stimulated WT macrophages but not macrophages deficient in Mac-1 or gp91 (the catalytic subunit of NOX2) to generate extracellular superoxide and iROS (Fig. 6A, 6B). Second, TLR3<sup>−/−</sup> and WT macrophages released the same amount of extracellular superoxide upon poly I:C challenge (Fig. 6F). Third, the genetic deletion or the pharmacological inhibition of NOX2 reduced poly I:C–elicited proinflammatory cytokine production (Fig. 6C, 6D). Fourth, poly I:C–mediated production of proinflammatory cytokines in TLR3<sup>−/−</sup> macrophages was decreased by NOX2 inhibition (Fig. 6G). Last, the impaired activation of MAPKs and NF-κB in macrophages deficient in Mac-1 (Fig. 5B, 5C) or gp91 (Fig. 6E) further implies that Mac-1 can induce inflammatory signaling through NOX2–induced iROS. Indeed, growing evidence has indicated an important role for iROS in inducing cellular immune responses to viral infection (50–52). Two independent groups showed recently that, in airway epithelial cells treated with either poly I:C or respiratory syncytial virus, NADPH oxidase is the major source of iROS and plays a crucial role in mediating downstream cellular immune responses (53, 54). In this article, we reported an important role for NOX2–associated ROS in dsRNA-mediated innate immune responses in macrophages.

In summary, the current study demonstrated that Mac-1 acts as a novel signaling PRR on the cell surface, sensing extracellular dsRNA. We further elucidate that poly I:C activates inflammatory oxidative enzyme NOX2 to produce ROS and to participate in the induction of proinflammatory cytokines in a TLR3-independent, but Mac-1–dependent manner. Through facilitating the internalization of poly I:C, Mac-1 activation also amplifies TLR3–dependent proinflammatory responses to dsRNA. Our results provide new insight into how macrophages recognize extracellular signals.
associated with lytic virus infections and identify a potential therapeutic target for virus-related inflammatory diseases.

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Disclosures

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References


