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*J Immunol* 2014; 193:1931-1941; Prepublished online 11 July 2014;
doi: 10.4049/jimmunol.1302863
http://www.jimmunol.org/content/193/4/1931

Supplementary Material  http://www.jimmunol.org/content/suppl/2014/07/11/jimmunol.1302863.DCSupplemental

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Differential Role for p120-Catenin in Regulation of TLR4 Signaling in Macrophages

Zhiyong Yang,*† Dong Sun,*†‡ Zhibo Yan,*† Albert B. Reynolds,§ John W. Christman,* Richard D. Minshall,*†‡ Asrar B. Malik,* Yang Zhang,* and Guochang Hu*†‡

Activation of TLR signaling through recognition of pathogen-associated molecular patterns is essential for the innate immune response against bacterial and viral infections. We have shown that p120-catenin (p120) suppresses TLR4-mediated NF-κB signaling in LPS-challenged endothelial cells. In this article, we report that p120 differentially regulates LPS/TLR4 signaling in mouse bone marrow–derived macrophages. We observed that p120 inhibited MyD88-dependent NF-κB activation and release of TNF-α and IL-6, but enhanced TIR domain–containing adapter-inducing IFN-β–dependent IFN regulatory factor 3 activation and release of IFN-β upon LPS exposure. p120 silencing diminished LPS-induced TLR4 internalization, whereas genetic and pharmacological inhibition of RhoA GTPase rescued the decrease in endocytosis of TLR4 and TLR4-MyD88 signaling, and reversed the increase in TLR4–TIR domain–containing adapter-inducing IFN-β signaling induced by p120 depletion. Furthermore, we demonstrated that altered p120 expression in macrophages regulates the inflammatory phenotype of LPS-induced acute lung injury. These results indicate that p120 functions as a differential regulator of TLR4 signaling pathways by facilitating TLR4 endocytic trafficking in macrophages, and support a novel role for p120 in influencing the macrophages in the lung inflammatory response to endotoxin. The Journal of Immunology, 2014, 193: 1931–1941.

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Received for publication October 24, 2013. Accepted for publication June 10, 2014.

This work was supported by National Institutes of Health National Heart, Lung, and Blood Institute Grant HL104092 (to G.H.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AM, alveolar macrophage; BAL, bronchoalveolar lavage; BMDM, bone marrow–derived macrophage; DN RhA, dominant negative mutant RhoA T19N adenovirus; EEA1, early endosomal Ag 1; ELW, extravascular lung water; IRAK, IL-1R–associated kinase; IRF, IFN regulatory factor; KC, keratinocyte-derived cytokine; LTA, lipoteichoic acid; p120, p120-catenin; poly(LC), polyninosinic-polycytidylic acid; RIPA, radioimmunoprecipitation assay; ROCK, Rho-associated kinase; siRNA, small interfering RNA; TRAF, TNFR-associated factor; TRIF, TIR domain–containing adapter-inducing IFN-β.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1302863

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plasma membrane to endosomes in LPS-stimulated macrophages. Mechanistically, inhibition of RhoA activation is required for p120-mediated TLR4 internalization. Our data indicate that p120 has a novel role in mediating macrophage involvement of inflammatory signaling.

Materials and Methods

Reagents

LPS (E. coli. 0111:B4) was from Calbiochem. Anti-TLR4 (M300), -TLR2, -MyD88, –IkBα, –early endosomal Ag 1 (EEA1), -IRAK1, –phospho-IRAK1, -TRAF6, -RhoA, -p120, and -HRP–conjugated Abs were obtained from Santa Cruz Biotechnology. Anti-IRF3, -pIRF3 (S396), and -IRAK1, -TRAF6, -RhoA, -p120, and -HRP–conjugated Abs were obtained from Biolegend and PBL Biomedical Laboratories. Rhodamine-BND protein agarose beads were from Santa Cruz Biotechnology. Amxaa cell line nucleofector kit V was obtained from Lonza. Cell-surface protein isolation kit and radioimmunoprecipitation assay (RIPA) buffer were from Pierce Biotechnology. Bicinchoninic acid kits and sample buffer were from Bio-Rad. Alexa Fluor 488– and Alexa Fluor 568–conjugated Abs were from Invitrogen. DMEM/F12 and nonenzymatic cell dissociation solution were from Cellgro. BSA, FBS, and heat-inactivated FBS were from Life Technologies. Dominant negative mutant RhoA T19N adenovirus (DN RhoA) was a gift from Dr. Jingsong Xu (University of Illinois at Chicago).

Animals

Seventy-four male C57BL/6 J mice (25–30 g) were used in this study. Mice were housed in microisolator cages under specific pathogen-free conditions, fed with autoclaved food, and used in experiments at 8–12 wk of age. Animal protocols received institutional review and committee approval, and all studies were conducted under anesthesia using either inhaled isoflurane or i.p.-injected ketamine (60 mg/kg).

Isolation and culture of mouse bone marrow–derived macrophages

Murine bone marrow–derived macrophages (BMDMs) were generated from femurs of C57BL/6J mice as described previously (12). In brief, mice were sacrificed by rapid cervical dislocation. Bone marrow was flushed from femurs using cell culture medium with 1% fetal bovine serum (FBS) and 10% heat-inactivated FBS, and cells were centrifuged at 2000 g for 5 min. Cell suspensions were resuspended in macrophage complete medium (DMEM/F12 with 10% FBS, 20% L929 cells conditioned medium, 10 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin). Cells (4 × 10^6) were then added to plastic petri dishes in 10 ml macrophage complete medium and incubated at 37°C and 5% CO2. On day 3, another 5 ml macrophage complete medium was added to each dish. After 7 d in culture, the adherent cells were ~95% pure macrophages, as evidenced by their expression of cell-surface markers HLA-DR (Biologicl), CD11b, and CD206, and these cells were used in subsequent experiments.

p120 knockdown in macrophages

p120 small interfering RNA (siRNA), which is a pool of 3 target-specific 20-nt siRNAs (Drhamcon) at a concentration of 50 nM, was added to 2 × 10^6 BMDMs in 6-well plates to deplete p120 using the protocol provided by the manufacturer. Successful downregulation of p120 in BMDMs in all experiments was confirmed by Western blot analysis. All experiments were performed 48 h posttransfection.

Electrotransfection

Transient transfection of BMDMs was performed according to the manufacturer’s instructions (Drhamcon). In brief, 2 × 10^6 BMDMs were resuspended in 20 μl nucleofector solution, mixed with 2 μg cDNA or 10 μM cDNA nontargeting or gene-specific ON-TARGETplus SMARTpool siRNAs. Nucleofection was performed using program D-032. Cells were rapidly transferred to pre-equilibrated culture medium and incubated at 37°C for 24–96 h. Viability of cells was evaluated by vital dye exclusion. Successful transfection was confirmed by Western blot analysis.

Flow cytometry

Adherent BMDMs were incubated with or without LPS (100 ng/ml) in medium for 30 min and detached with nonenzymatic cell dissociation solution. Cell suspensions were centrifuged for 5 min at 200 × g and washed twice with cold PBS with 1% BSA and without Ca^2+ and Mg^2+. The pellets were resuspended in ice-cold PBS with 1% BSA and without Ca^2+ and Mg^2+ at a final cell concentration of 2 × 10^6/ml. Cell suspensions (1 × 10^6 at 50 μl) were added to each tube and incubated with 0.5 μg anti-mouse CD16/CD32 Abs for 20 min on ice. BMDMs were then incubated with PE-conjugated anti-TLR4 Abs or mouse IgG1 isotype control (0.5 μg/tube) for 45 min on ice, washed, and analyzed by flow cytometry (Becton Dickinson LSR I).

RhoA-GTP pull-down assay

RhoA pull-down assays were performed using Rhodamine-BND Protein GST Beads (Cytoskeleton) according to the manufacturer’s instructions. Cells were washed with ice-cold PBS and lysed with cell lysis buffer (25 mM Tris, pH 7.5, 10 mM MgCl2, 300 mM NaCl, 2% octylphenyl-polyethylene glycol, and protease inhibitor). Lysates were centrifuged at 10,000 × g for 5 min, and the supernatant was incubated with 60 μg Rhodamine-BND Protein GST Beads for 1 h at 4°C on a roller system. The beads were then collected by centrifugation at 5000 × g, 4°C for 1 min, and washed twice with wash buffer (25 mM Tris, pH 7.5, 30 mM MgCl2, 40 mM NaCl). Beads were finally resuspended in Laemml sample buffer (30 μl) and boiled for immunoblotting with anti-RhoA Ab. The total level of RhoA was measured by Western blot analysis of cell lysates.

Immunofluorescence

Immunofluorescence was performed as previously described (13). Cells plated on coverslips were fixed with 2% paraformaldehyde for 15 min, washed 3 times with 100 μM glycine in HBSS for 10 min, followed by washing once with HBSS for 10 min. Cells were permeabilized with 0.1% Triton X-100 in HBSS for 30 min and then incubated with anti-TLR4 (1:100) or anti-EEA1 (1:200) Ab for 1 h, followed by incubation with Alexa Fluor 568– or Alexa Fluor 488–conjugated secondary Abs for 1 h. Cell nuclei were stained with DAPI (1:2000) for 15 min. The cells were rinsed three times and finally mounted on glass slides using ProLong antifade mounting medium (Molecular Probes). Confocal images were acquired with a laser-scanning confocal microscope (Zeiss LSM 510 META) using Hg lamp and UV-filter set to detect DAPI (band pass 385–470 nm emission), 488 nm excitation laser line to Alexa 488 (band pass 505–550 nm emission), and 568 nm excitation laser line to Alexa Fluor 568 (excitation/emission ~578/603 nm). Optical sections had a thickness <1 μm (pinhole set to achieve 1 Airy unit).

Western blotting and immunoprecipitation

Cells were lysed in RIPA buffer supplemented with 1 mM PMSF, 1 mM Na3VO4, protease, and phosphatase inhibitor mixture. Lysates were sonicated and centrifuged at 10,000 × g for 10 min at 4°C. The concentration of lysates was measured by protein assay. Equal amounts of proteins were loaded for PAGE (10–15%) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and probed with primary Abs for 2 h at room temperature or overnight at 4°C and then incubated with HRP-conjugated secondary Abs at room temperature for 1 h. The protein bands were determined using the ECL reagent (Pierce). Relative band densities of the various proteins were measured from scanned films using ImageJ Software (National Institutes of Health).

Immunoprecipitation analysis was performed as described previously (11, 13). Cell lysates were precleared with 1 μg normal rabbit IgG and 20 μl protein A+G agarose beads for 2 h at 4°C, and then incubated overnight at 4°C with primary Ab, followed by addition of 25 μl Protein A/G PLUS-Agarose and further incubated at 4°C for 2 h. Equal amounts of proteins were electrophoresed on SDS-PAGE gels (10–12%) and subsequently transferred to 0.22-mm nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and probed with the appropriate Abs.

IRF3 dimerization assay

Cell lysates were electrophoresed on 10% native PAGE gels. IRF3 monomers and dimers were detected by Western blot analysis.

Cell-surface biotinylation

Cell-surface biotinylation was used for TLR4 endocytosis assay according to a method described in detail previously (14) with slight modifications. BMDMs were washed three times with ice-cold PBS and then incubated in sulfo-NHS-SS-biotin solution for 30 min at 4°C. At this point in the protocol, biotinylated TLR4 proteins reside within the endosomal compart-
ment. Subsequently, the cells were lysed, and the biotinylated proteins were isolated by streptavidin-agarose beads, eluted into SDS sample buffer, and separated by 10% SDS-PAGE. Biotinylated TLR4 was analyzed by Western blot analysis as described earlier.

Depletion of alveolar macrophages in mice

Depletion of alveolar macrophages (AMs) was performed as described previously (15). The clodronate liposome (Encapsula NanoSciences LLC, Nashville, TN) was delivered to anesthetized (ketamine 90 mg/kg i.p.) mice by nebulization.

Murine model of LPS-induced acute lung injury

BMDMs were isolated and cultured using a standard protocol as previously described (16). BMDMs were transfected with a scrambled siRNA or p120 siRNA. AM-depleted mice were divided into three groups (n = 6 each). Saline or BMDMs transfected with a scrambled siRNA or p120 siRNA (2 × 10⁶ cells, 200 μl total volume each) were given to AM-depleted mice via a jugular venous cannula 30 min before LPS challenge. Mice were then nebulized and challenged with 1.5 mg/ml LPS for 1 h. At 4 h after LPS challenge, mice were sacrificed and lung injury was assessed by analysis of bronchoalveolar lavage (BAL) fluid, extravascular lung water (ELW) measurement, and biochemical/immunological analysis of lung tissue.

Determination of polymorphonuclear neutrophil counts in BAL fluid

At the end of the experiments, BAL was performed by intratracheal injection of 1 ml PBS followed by gentle aspiration. The lavage was repeated three times. The pooled BAL fluid was centrifuged, and cell pellets were suspended in PBS. Cell suspensions were cytospun onto slides with a cytocentrifuge (Shandon, Southern Sewickley, PA). Slides were stained with Diff-Quick dye (Dade Behring, Newark, DE) and examined at a magnification of ×20 and ×40 by light microscopy. The percentage of polymorphonuclear neutrophils was determined after counting 300 cells in randomly selected fields.

FIGURE 1. Silencing of p120 expression potentiates the LPS-initiated, MyD88- mediated TLR4 signaling pathway in macrophages. BMDMs were transfected with a scrambled (Sc) siRNA or p120 siRNA. After 48 h, the cells were treated with 100 ng/ml LPS for 12 h. (A) Effects of p120 knockdown on the association of TLR4 and MyD88. Immunoprecipitation of TLR4 and immunoblotting with Ab against MyD88 were performed. The association of MyD88 and TLR4 was augmented in p120 knockdown macrophages. (B) Effects of p120 knockdown on degradation of IκB-α after LPS challenge. (C) TNF-α level in supernatants was measured by ELISA. Data are mean ± SEM of three independent experiments. *p < 0.05 compared with control group (without LPS treatment), †p < 0.05 compared with Sc siRNA group. ND, not detectable.

FIGURE 2. Silencing of p120 expression suppresses the LPS-initiated, TRIF-mediated TLR4 signaling pathway in macrophages. BMDMs were transfected with a scrambled (Sc) siRNA or p120 siRNA. After 48 h, the cells were treated with 100 ng/ml LPS for 4 h. (A) Effects of p120 knockdown on the association of TLR4 and TRIF. Immunoprecipitation of TLR4 and immunoblotting with Ab against TRIF were performed. The association of MyD88 and TRIF was diminished in p120 knockdown macrophages. (B) Effects of p120 knockdown on IRF3 phosphorylation after LPS challenge. (C) Effects of p120 knockdown on IRF3 dimerization after LPS challenge. Cells lysates were subjected to native PAGE, and monomeric and dimeric IRF3 were detected with anti-IRF-3 mAb. (D) IFN-β level in supernatants was measured by ELISA. Data are mean ± SEM of three independent experiments. *p < 0.05 compared with control group (without LPS treatment), †p < 0.05 compared with Sc siRNA group. ND, not detectable.
Assessment of pulmonary edema formation
ELW was used as an index of lung water content and edema (17).

Statistical analysis
One-way ANOVA and Student Newman–Keuls test for post hoc comparisons were used to determine differences between control and experimental groups. Parameter changes between different groups over time were evaluated by a two-way ANOVA with repeated measures. Data are expressed as mean ± SEM. Differences were considered significant when \( p < 0.05 \).

Results
p120 suppresses MyD88-dependent TLR4 signaling in macrophages
To address the potential mechanisms by which p120 regulates MyD88-dependent TLR4 signaling, we first determined whether p120 associated with TLR4 and its downstream molecules. Surprisingly, we did not detect p120 association with TLR4, MyD88, IRAK1, and TRAF6 in either the absence or the presence of LPS stimulation (Supplemental Fig. 1A). Recruitment of MyD88 to TLR4 is one of the earliest events of TLR4 signaling (1, 2, 6). We previously showed that p120 inhibited TLR4 signaling by attenuating the association of MyD88 and TLR4 in lung endothelial cells, and that p120 degradation in response to LPS challenge led to an increase in TLR4 signaling (11). In this study, we investigated the potential role of p120 in the regulation of TLR4 signaling in macrophages. As expected, LPS caused a transient increase in the association between TLR4 and MyD88. Remarkably, knockdown (90%) of p120 augmented the LPS-induced association of TLR4 and MyD88, whereas p120 siRNA alone had no effect on this interaction (Fig. 1A). LPS also induced IRAK-1 phosphorylation and degradation, consistent with previous studies (2), whereas these responses were exaggerated by p120 silencing (Supplemental Fig. 1B). Furthermore, activation of NF-κB as measured by LPS-induced degradation of inhibitory IκB-α subunit was consistently increased in macrophages transfected with p120 siRNA (Fig. 1B). Next, we studied the impact of p120 depletion on production of the MyD88-dependent cytokine TNF-α and IL-6 (18, 19). In response to LPS challenge, p120 knockdown led to substantially greater production of TNF-α (Fig. 1C) and IL-6 (Fig. 1D) compared with scrambled siRNA-treated macrophages, whereas p120 knockdown alone had no effect on the production of TNF-α and IL-6. To determine whether p120 specifically inhibits TLR signaling, we used a non-TLR control curdlan (20) to stimulate macrophages. Curdlan dramatically caused the release of TNF-α and IL-6, whereas these responses were not altered by p120 knockdown (Supplemental Fig. 2). These findings indicate that p120 is a potential negative regulator of MyD88-mediated TLR4 signaling in macrophages.

p120 augments TRIF-dependent TLR4 signaling in macrophages
To discern whether p120 expression regulates the TLR4-triggered TRIF pathway, we examined LPS-induced recruitment of TRIF to TLR4 and subsequent activation of the TRIF–IRF3–IFN-β signaling axis (2, 6). LPS induced the recruitment of TRIF to TLR4 within 15–30 min in scrambled siRNA-treated macrophages. In contrast with the data shown earlier for the MyD88 pathway, p120 knockdown nearly abolished LPS-induced association between TLR4 and TRIF (Fig. 2A). To confirm these data, we interrogated the requirement of TRIF recruitment to TLR4 for activation of IRF3 that requires the phosphorylation and dimerization of the

FIGURE 3. Knockdown of p120 has no effect on TLR2/3-mediated cytokine production. (A) BMDMs were transfected with a scrambled (Sc) or p120 siRNA. After 48 h, effective knockdown of p120 expression was confirmed by Western blotting. (B–G) BMDMs transfected with a Sc or p120 siRNA were stimulated with 5 μg/ml LTA (B–D) or 20 μg/ml poly(I:C) (E–G) for 12 h. TNF-α, IL-6, and IFN-β in the supernatants were measured by ELISA. ND, not detectable.
monomer form of IRF3 (21, 22). LPS markedly induced the phosphorylation of IRF3 in macrophages transfected with a scrambled siRNA, whereas LPS-induced IRF3 phosphorylation was abolished in p120 siRNA-treated macrophages (Fig. 2B). Significantly, comparable intensities of IRF3 bands were detected (Fig. 2B), indicating that the differences in IRF3 phosphorylation were not due to variations in the total level of IRF3 expression. Consistently, IRF3 dimers were increased after LPS treatment, whereas depletion of p120 inhibited LPS-induced IRF3 dimerization (Fig. 2C). To determine the impact of p120 depletion on IRF3 transactivation, we analyzed LPS-mediated induction of TRIF-controlled, IRF3-dependent IFN-β production (Fig. 2D). Depletion of p120 with a specific siRNA significantly decreased LPS-driven production of IFN-β (Fig. 2D) compared with the responses observed in scrambled siRNA-transfected cells. p120 knockdown alone had no effect on IFN-β production (Fig. 2D). These data indicate that p120 increased TRIF-mediated TLR4 signaling in macrophages.

p120 is unable to regulate TLR2/3 signaling

To further determine whether the differential effect of p120 on MyD88 and TRIF signaling was specific for TLR4, we examined the effects of p120 depletion on TLR2/TLR3 signaling as measured by the production of TLR-induced cytokines, including TNF-α, IL-6, and IFN-β. As shown in Fig. 3A, p120 protein was decreased 90% by transfection of p120-specific siRNA, as measured by immunoblotting. However, although TNF-α, IL-6, and IFN-β secretion increased significantly after treatment of BMDMs with TLR2 ligand LTA, p120 knockdown had no effect on the production of TNF-α, IL-6, and IFN-β in response to LTA stimulation (Fig. 3B–D). Similarly, the release of TNF-α, IL-6, and IFN-β upon stimulation of TLR3 with the synthetic ligand poly(I:C) in scrambled siRNA-treated macrophages was not significantly affected by p120 siRNA transfection (Fig. 3E–G).

p120 facilitates LPS-induced TLR4 internalization

TLR4 internalization in response to LPS stimulation has been shown to regulate LPS-induced proinflammatory signaling (5, 6, 23). Therefore, we tested whether p120 regulates TLR4 internalization in macrophages. Western blot analysis of surface biotinylated TLR4 indicated that LPS caused a gradual increase in TLR4 internalization within 30 min in scrambled siRNA-treated macrophages. However, p120 knockdown significantly reduced LPS-induced TLR4 internalization (Fig. 4A, 4B). These data were confirmed by surface staining of macrophages and analysis by

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**FIGURE 4.** Silencing of p120 expression inhibits LPS-induced endocytosis of TLR4. BMDMs were transfected with a scrambled (Sc) siRNA or p120 siRNA. After 48 h, the cells were treated with 100 ng/ml LPS for the indicated times. (A) Cell-surface biotinylation of TLR4 in BMDMs. BMDMs were labeled with sulfo-NHS-SS-biotin. Cells were lysed with RIPA buffer, and the cell lysates were either used directly for Western blot analysis or precipitated with streptavidin-conjugated agarose for biotinylated cell-surface proteins and analyzed with Western blotting with TLR4-specific Abs. Results are representative of three independent experiments. (B) Relative densities of the bands of TLR4 protein expression. The density of TLR4 protein in the control Sc group (biotin) was used as a standard (1 arbitrary unit) to compare relative densities in the other groups. (C) Representative histograms of flow-cytometry experiments demonstrating the effects of p120 silencing on cell-surface expression of TLR4 protein in response to LPS stimulation. Cell-surface expression of TLR4 protein was evaluated using PE-conjugated MTS510 Ab and FACS analysis. Black lines depict staining with irrelevant IgG2a, blue lines for untreated cells, and red lines for LPS-treated cells. (D) Quantitative data showing changes in mean fluorescent intensity (MFI) of PE-TLR4 after LPS stimulation (n = 3). (E and F) Accumulation of TLR4 in the endosomal compartment of macrophages. After labeling plasma membrane TLR4, cells were incubated at 37°C in the presence of LPS for the indicated times (E) or 20 min (F). Internalized TLR4 was detected by immunofluorescence for EEA1 (green) and TLR4 (red) in control (E), Sc, or p120 siRNA-treated macrophages (F) after LPS stimulation. Scale bar, 50 μm. *p < 0.05 compared with corresponding Sc siRNA+LPS groups (B) or no LPS group (D), †p < 0.05 compared with Sc siRNA+LPS group (D).
flow cytometry using a PE-conjugated anti-TLR4 Ab. Consistent with other reports (5, 6, 23), within 30 min of LPS treatment, TLR4 cell-surface staining decreased in macrophages transfected with a scrambled siRNA, indicating that TLR4 is internalized after ligand binding. In contrast, depletion of p120 gene expression with a specific siRNA prevented the LPS-induced internalization of TLR4 (Fig. 4C, 4D). Furthermore, the localization of TLR4 to early endosomes was confirmed by staining fixed cells with an Ab to the early endosomal marker EEA1 (Fig. 4E). In unstimulated cells, TLR4 was present on small EEA1+ endosomes that grew significantly larger after LPS stimulation (Fig. 4E). However, p120 knockdown significantly reduced the amount of TLR4 in EEA1+ endosomes compared with scrambled siRNA-treated cells (Fig. 4F). To determine whether p120 also regulates TLR2 internalization in macrophages, we observed the effects of p120 knockdown on TLR2 endocytosis in response to LTA. As expected, LTA induced a gradual increase in TLR2 internalization within 30 min in scrambled siRNA-treated macrophages, whereas

**FIGURE 5.** Genetic and pharmacological inhibition of RhoA rescues LPS-induced endocytosis of TLR4 impaired by p120 depletion. BMDMs were transfected with a scrambled (Sc, B) or p120 siRNA (B–K), or a DN RhoA cDNA or vector (Vec) (C–G). Transfected BMDMs were exposed to 100 ng/ml LPS for 15 min. The activation of RhoA was measured by RhoA-GTP pull-down assay (B and C). In some experiments, after p120 depletion, BMDMs pretreated with 10 μM Y27632 or vehicle for 30 min were exposed to 100 ng/ml LPS for the indicated times (H–K). (A) Association of p120 and RhoA in macrophages in the absence and presence of LPS. (B) Effects of LPS on RhoA activation in BMDMs transfected with a Sc or p120 siRNA. (C) Effects of exogenous expression of DN RhoA on LPS-induced RhoA activation. (D) Effects of exogenous expression of DN RhoA on cell-surface biotinylation of TLR4 in p120 silencing BMDMs. Representative histograms of flow cytometry experiments demonstrating the effects of RhoA activity on cell-surface expression of TLR4 protein in p120 silencing cells in response to LPS stimulation. Cell-surface expression of TLR4 protein was evaluated using PE-conjugated MTS510 Ab and FACS analysis. (E) Quantitative data showing changes in mean fluorescent intensity (MFI) of PE-TLR4 (n = 3). (F) Effects of RhoA activity on cell-surface biotinylation of TLR4 in p120 silencing BMDMs. BMDMs were labeled with sulfo-NHS-SS-biotin. Cells were lysed with RIPA buffer and the cell lysates were either used directly for Western blot analysis or precipitated with streptavidin-conjugated agarose for biotinylated cell-surface proteins and analyzed with Western blots with TLR4-specific Abs. Results are representative of three independent experiments. (G) Relative densities of the bands of TLR4 protein expression (F). The density of TLR4 protein in the control Vec group (Biotin) was used as a standard (1 arbitrary unit) to compare relative densities in the other groups. (H) Effects of Y27632 on cell-surface biotinylation of TLR4 in p120 silencing BMDMs. Representative flow cytometry data showing TLR4 expression on the surface of cells. (I) Quantitative data showing changes in mean fluorescent intensity (MFI) of PE-TLR4 (n = 3). (J) Effects of Y27632 on cell-surface biotinylation of TLR4 in p120 silencing BMDMs. Results are representative of three independent experiments. (K) Relative densities of the bands of TLR4 protein expression (J). *p < 0.05 compared with Vec group (E), corresponding p120 siRNA+Vec group (G), no LPS group (I), or corresponding p120 siRNA alone group (K); †p < 0.05 compared with LPS+Vec group (E) or LPS group (I). B, biotin; CON, control; DRA, DN RhoA.
p120 knockdown did not alter LTA-induced TLR2 internalization (Supplemental Fig. 3). Taken all together, our data show that p120 specifically facilitates TLR4 trafficking from the cell surfaces to early endosomes.

RhoA mediates p120-induced TLR4 internalization after LPS stimulation

Endocytosis of TLR4 after LPS stimulation is dependent on dynamin and clathrin (5). Actin polymerization and depolymerization are required to regulate endocytosis in mammalian cell lines (24, 25). Rho inhibits clathrin-mediated endocytosis of membrane proteins (26). We therefore examined whether the regulatory role for p120 in LPS-induced TLR4 internalization is mediated via the small GTPase RhoA, which is known to be critical for active reorganization of cellular actin (27). Previous study has indicated that p120 negatively regulates RhoA activation via its direct or indirect interaction with RhoA-GDP and subsequent inhibition of GDP dissociation (27, 28). In this study, we showed that p120 associated with RhoA in macrophages, and this association was significantly attenuated after LPS challenge (Fig. 5A). LPS caused RhoA activation in scrambled siRNA-treated BMDMs, whereas this response was significantly increased in p120 siRNA-treated cells (Fig. 5B). We next infected p120 siRNA-treated BMDMs with adenoviral vectors expressing DN RhoA. The activity of RhoA was nearly abrogated by transfection of the dominant negative form of the GTPase (Fig. 5C). Importantly, in p120-depleted macrophages, exogenous expression of empty vector or DN RhoA had no effect on the surface expression of TLR4, whereas impaired internalization of TLR4 by depletion of p120 in the presence of LPS could be rescued by expression of DN RhoA (Fig. 5D, 5E). This finding was confirmed in cell-surface TLR4 biotinylation and internalization studies (Fig. 5F, 5G).

Because RhoA is known to activate Rho kinase, we assessed the effect of the Rho kinase inhibitor Y27632 on TLR4 endocytosis upon LPS exposure. Inhibition of the activity of downstream RhoA effectors Rho-associated kinases (ROCKs) I and II with Y27632 led to a restoration of impaired TLR4 internalization in p120 knockdown macrophages after LPS stimulation (Fig. 5H–K), validating the finding that RhoA may regulate p120-mediated TLR4 endocytosis in macrophages after LPS stimulation. Taken all together, these results indicate that p120 regulates LPS-induced TLR4 endocytosis via modulation of RhoA GTPase activity.

RhoA contributes to the regulatory role of p120 in TLR4 signaling

To investigate whether RhoA is involved in p120-dependent regulation of TLR4 signaling after LPS stimulation, we examined the effect of RhoA inhibition on the association of TLR4 with its adaptor proteins MyD88 and TRIF in p120 siRNA-treated macrophages. Effective transient siRNA knockdown of p120 (90%) and exogenous expression of DN RhoA were confirmed by Western blot analysis (data not shown). As shown in Fig. 1A, LPS caused a robust increase in the association between TLR4 and MyD88 in p120-depleted BMDMs expressing empty vector, whereas expression of DN RhoA reduced LPS-induced association between TLR4 and MyD88 (Fig. 6A). Furthermore, increased degradation of inhibitory IκB-α subunit upon LPS exposure was consistently abolished by expression of DN RhoA in macrophages transfected with p120 siRNA (Fig. 6B), and the increased TNF-α production in response to LPS challenge in p120 siRNA-treated BMDMs expressing empty vector was also reversed by expression of DN RhoA (Fig. 6C). We also observed that treatment with a pharmacological inhibitor of RhoA reversed increased association of TLR4 and MyD88 (Fig. 6D), IκB-α degradation (Fig. 6E), and TNF-α production (Fig. 6F) after LPS stimulation in p120 siRNA-treated BMDMs.

In contrast with the role of RhoA in MyD88-mediated TLR4 signaling, genetic and pharmacological inhibition of RhoA blocked the decrease in the association of TLR4 and TRIF (Fig. 7A, 7B), IRF3 phosphorylation (Fig. 7C, 7D), and IFN-β production (Fig. 7E, 7F) after LPS stimulation in p120 siRNA-treated BMDMs. Collectively, these findings suggest that the inactivation of RhoA rescues LPS-induced TLR4 signaling impaired by p120 depletion, suggesting a central role in mediating the disparate effects of p120 on the MyD88 and TRIF signaling pathways.

Reduced expression of p120 in macrophages enhances LPS-induced acute lung injury

Our results thus far indicated that p120 knockdown in macrophages caused increased MyD88-dependent and decreased TRIF-dependent cytokine responses activated by TLR4. These findings raise the possibility that the intentional lowering of p120 levels in lung macrophages would result in accentuated acute lung inflammation and secondary injury through impacting TLR4 signaling (11, 29). We therefore determined whether a change in surface expression of TLR4 controlled by p120 could influence
the inflammatory response using an in vivo model. Mice were depleted of AMs by using a liposomal clodronate technique (15). To ensure that clodronate reduced macrophage numbers, we performed BAL on mice administered clodronate, as well as PBS, and counted a minimum of 500 cells/slide. Lavageable AM count was reduced by 75% at 4 d with a 10 ml aerosolized dose of 20 mg/ml clodronate liposome solution. p120 expression was downregulated ∼90% in BMDMs transfected with a specific p120 siRNA (Fig. 8A). In vivo injection of BMDMs treated with a scrambled siRNA or p120 siRNA resulted in comparable reconstitution of macrophages in the lung (Supplemental Fig. 4). The total neutrophil count in BAL fluid and ELW increased ∼8-fold and 5-fold after administration of LPS in control mice (no AM depletion) and AM-depleted mice receiving scrambled siRNA-transfected BMDMs, respectively. Mice depleted of AMs before LPS challenge showed less PMN infiltration and lung edema formation. AM-depleted mice receiving p120 siRNA-transfected BMDMs showed a significant increase in ELW (Fig. 8B) and the neutrophil counts in BAL fluid (Fig. 8C) compared with mice receiving scrambled siRNA-transfected BMDMs.

Proinflammatory cytokines were also measured in the BAL fluid collected from these mice. We found that TNF-α, IL-6, and keratinocyte-derived cytokine (KC) were elevated in the BAL fluid in response to LPS challenge in control mice (no AM depletion). However, the levels of TNF-α, IL-6, and KC after LPS challenge were significantly attenuated in AM-depleted mice (Fig. 8D–F). AM-depleted mice receiving scrambled siRNA-transfected BMDMs showed variably increased levels of proinflammatory cytokines, whereas AM-depleted mice receiving p120-silenced BMDMs dramatically enhanced the levels of TNF-α, IL-6, and KC (Fig. 8D–F). In contrast, AM-depleted mice receiving p120-silenced BMDMs had a remarkably reduced level of IFN-β compared with those mice receiving scrambled siRNA-transfected BMDMs (Fig. 8G).

Discussion

Prolonged and uncontrolled inflammation in severe sepsis is a major contributor to morbidity and mortality. Tight regulation of TLR4 signaling is a pivotal mechanism to maintain the auspicious balance between proinflammatory and anti-inflammatory immune responses, which determines the intensity and duration of the inflammatory response, which influences the progression and clinical outcome of severe sepsis. To date, many regulators that reduce or terminate the activation of TLR4 signaling pathways have been identified that operate via various mechanisms that include dissociation of adaptor complexes, degradation and competition of signal proteins, and regulation of transcription factors (30, 31). Our study shows a relatively simple but novel mechanism of TLR4 regulation that p120 differentially and specifically regulates TLR4 signaling in macrophages via modulation of TLR4 internalization. p120 inhibits MyD88-mediated NF-κB activation and generation of proinflammatory cytokine TNF-α, but enhances TRIF-mediated IRF3 activation and IFN-β production. Importantly, modification of cell-surface TLR4 expression via p120 knockdown in macrophages exaggerates LPS-induced lung injury, indicating that p120 may be a potential therapeutic target for treatment of inflammatory diseases such as acute respiratory distress syndrome in humans.

Endocytosis of TLR4 and subsequent trafficking through the endosomal system is indispensable for the regulation of innate immunity and inflammatory response during sepsis. Upon LPS stimulation, TLR4 interacts with CD14 and MD2 on the plasma membrane where MyD88-mediated signaling is activated to initiate NF-κB activation and inflammatory cytokine expression, and then TLR4 is transported to endosomes where TRIF-mediated signaling can lead to IRF3 activation and the expression of IFNs (22, 32–34). Internalized TLR4 can be delivered to recycling endosomes and Golgi for recycling or to lysosomes for degradation (5, 35–37). Inhibition of TLR4 endocytosis increased LPS-induced
NF-κB activation and proinflammatory signaling, whereas disruption of TLR4 endocytosis was shown to inhibit TRIF-dependent TLR4 signaling upon LPS stimulation, indicating that the endocytosis of TLR4 is an important mechanism for termination of MyD88-mediated TLR4 signaling (6). Our results also show that p120 promotes the endocytosis of TLR4 in response to LPS stimulation, which, in turn, suppresses MyD88-mediated TLR4 signaling and release of the proinflammatory cytokine TNF-α. In contrast, p120 stimulates TRIF-mediated TLR4 signaling and IFN-β production via enhancement of endocytosis of TLR4. These data support the conclusion that p120 differentially regulates LPS-induced RhoA activation, verifying the inhibitory effect of p120 on RhoA activity in macrophages.

The mechanism by which p120 promotes the endocytosis of TLR4 in response to LPS stimulation remains unclear. The endocytosis of TLR4 is dependent on dynamin and clathrin (5, 6). Furthermore, clathrin-mediated endocytosis can be negatively regulated by Rho signaling (26, 38), and p120 has been shown to inhibit RhoA activation (39). These findings raise the possibility that p120 facilitates TLR4 internalization via suppression of RhoA activation in macrophages. Our results indicate that p120 silencing significantly increases LPS-induced RhoA activation, verifying the inhibitory effect of p120 on RhoA activity in macrophages. It has been reported that, in response to TLR2/3 stimulation, RhoA is required for NF-κB activation but is dispensable for type I IFN generation (40). Using genetic and pharmacological inhibitors, we demonstrated that inhibition of RhoA reversed p120 silencing–mediated increase in MyD88-mediated TLR4 signaling, NF-κB activation, and TNF-α production, as well as the decrease in TRIF-mediated TLR4 signaling, IRF3 activation, and IFN-β production. These data also
indicate that RhoA serves as a negative regulator of TLR4 internalization in response to LPS stimulation in macrophages. RhoA can regulate the actin cytoskeleton through multiple signaling pathways, such as the ROCKI/myosin L chain/Myosin II pathway, ROCKI/LIM-kinase/cofilin pathway, and mDia/Src pathway (41). This study showed that Y27632, a ROCKI inhibitor, rescued LPS-induced TLR4 endocytosis impaired by p120 knockdown, suggesting that p120 regulates the endocytosis of TLR4 in macrophages via the small GTPase RhoA and its downstream effector Rho kinase.

The role of p120 expression in macrophages during an inflammatory response such as that evoked by bacterial sepsis has not been reported. In this study, we used a well-established mouse model of LPS-induced acute lung injury (42) to examine the effect of macrophages with varying levels of p120 expression on lung inflammatory injury. We found that i.v. administration of p120 siRNA-transfected BMDMs caused robust lung inflammation and edema in LPS-challenged mice. Our data indicate that p120 expression in macrophages plays an important role in LPS-induced lung inflammatory injury. These results, together with our previous findings from pulmonary endothelial cells (11), strongly highlight the fact that p120 expression in both endothelial cells and macrophages is important in the regulation of innate immunity and inflammation during sepsis.

Our observation that the cytokine (TNF-α, IL-6, and IFN-β) response to TLR2/TLR3 stimulation was normal in the p120 knockdown macrophages suggests that the role of p120 in the regulation of MyD88- and TRIF-mediated TLR signaling is specific to TLR4. Like TLR4, TLR2 is found at the cell surface, whereas the TLR3 involved in the recognition of nucleic acids is localized within endolysosomal compartments (43). Interestingly, in contrast with TLR4, TLR2 internalization is clathrin independent and lipid raft dependent (44). Our data demonstrated that LTA-induced TLR2 internalization was not affected by silencing of p120, suggesting that p120 is unable to regulate TLR2 endocytosis and subsequent release of cytokines in response to LTA stimulation in macrophages. Taken together, p120 selectively regulates LPS/TLR4 signaling via RhoA-mediated TLR4 endocytosis in macrophages.

In summary, we demonstrate for the first time, to our knowledge, that p120 differentially and selectively regulates LPS-induced TLR4 signaling in macrophages. Our data support a model in which p120 promotes LPS-induced TLR4 internalization via inactivation of RhoA GTPase. By facilitating TLR4 internalization, p120 downregulates MyD88-mediated TLR4 signaling and proinflammatory cytokine production, whereas at the same time it upregulates TRIF-mediated TLR4 signaling and IFN-β production (Fig. 9). These findings add to our understanding of molecular mechanisms mediating anti-inflammatory modulation of immune responses by p120. Thus, targeting p120, or its downstream effectors RhoA and Rho kinase, may be an effective strategy for preventing acute lung inflammatory injury.

Acknowledgments
We thank Maricela Castellon (Department of Anesthesiology, University of Illinois College of Medicine) for technical assistance.

Disclosures
The authors have no financial conflicts of interest.

References