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Rac1 Negatively Regulates Lipopolysaccharide-Induced IL-23 p19 Expression in Human Macrophages and Dendritic Cells and NF-κB p65 trans Activation Plays a Novel Role

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IL-23 is a heterodimeric cytokine composed of a unique p19 subunit and of a p40 subunit that is also common to IL-12. We defined the distinct signaling mechanisms that regulate the LPS-mediated induction of IL-23 p19 and p40 in human macrophages and dendritic cells. We found that the overexpression of dominant-negative Rac1 (N17Rac1) enhanced LPS-induced IL-23 p19 expression but did not alter p40 expression or IL-12 p70 production in PMA-treated THP-1 macrophages and in human monocyte-derived dendritic cells. Although the inhibition of either p38 MAPK or JNK enhanced LPS-induced p19 expression, N17Rac1 did not influence either p38 MAPK or JNK activation. By contrast, N17Rac1 augmented both NF-κB gene expression and p65 trans activation stimulated by LPS without affecting the degradation of 1κB-α or DNA binding to NF-κB. Furthermore, small interference RNA of NF-κB p65 attenuated cellular amounts of p65 and suppressed LPS-induced p19 expression but did not affect p40 expression. Our findings indicate that Rac1 negatively controls LPS-induced IL-23 p19 expression through an NF-κB p65 trans activation-dependent, 1κB-independent pathway and that NF-κB p65 regulates LPS-induced IL-23 p19, but not p40, expression, which causes differences in the control of IL-23 p19 and p40 expression by Rac1. The Journal of Immunology, 2006, 177: 4550–4557.
through association with IkBs. Upon cellular stimulation, IkBs become phosphorylated, resulting in the degradation and liberation of Rel homodimers, which after translocation to the nucleus bind the DNA-regulatory element of target genes (10). In addition, p65 is responsible for the transcriptional activity of NF-κB, which is regulated by coactivators in the nucleus (11) and the phosphorylation of p65 that involves Ser^276 (12), Ser^529 (13), and Ser^326 (14).

The Rho family GTPase Rac1 is a key regulator of various cellular functions such as cytoskeletal reorganization, cellular growth, and apoptosis (15, 16). Although Rac1 can activate NF-κB in various types of cells (17–19), the role of Rac1 in NF-κB activation and cytokine expression in human macrophages and DCs has not been determined.

This study examines whether the Rac1 pathway is required for LPS-induced IL-23 mRNA expression in human macrophages and DCs. The results indicated that Rac1 negatively regulates expression of the IL-23 p19 subunit, but not that of p40 induced by LPS. Moreover, we investigated the molecular mechanism that underlies the regulation of IL-23 expression by Rac1, and found that the activation of Rac1 by LPS affects transcriptional activity of the NF-κB p65 subunit leading to the regulation of p19 mRNA expression.

Materials and Methods

Reagents

PMA and LPS (from Escherichia coli serotype 0111:B4) were obtained from Sigma-Aldrich. SB203580, SP600125, and Wortmannin were purchased from Calbiochem and BIOMOL, respectively. Total and phosphorylated p38, JNK, IkB-α, and NF-κB p65 were detected using Abs from Cell Signaling Technology.

Cell culture

Human monocytic THP-1 cells (American Type Culture Collection) in RPMI 1640 (Invitrogen/Life Technologies) with 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μM 2-ME supplemented with 10% FBS (Eoquech-Bio) were cultured under a humidified 5% CO2 atmosphere at 37°C. Phorbol esters induced this cell line to differentiate into macrophages as follows (20). The cells were washed and resuspended in fresh RPMI 1640 supplemented with 10% FBS and 50 U/ml penicillin G sodium, 50 μg/ml streptomycin sulfate (Invitrogen Life Technologies); complete medium) containing with 100 ng/ml PMA (21). After 48 h, the cells were washed three times and applied in subsequent experiments.

Monocyte-derived DCs (MD-DCs) were generated as described (22, 23). Briefly, PBMC were separated by density gradient centrifugation from leukocyte concentrates obtained from healthy volunteers. Monocytes purified from PBMC by positive selection with anti-CD14 magnetic beads (Miltenyi Biotec) were cultured in complete medium supplemented with 10 ng/ml human rGM-CSF (R&D Systems) and 10 ng/ml human rIL-4 (R&D Systems).

Every 2 days, 10 ng/ml human rGM-CSF (R&D Systems) and 10 ng/ml human rIL-4 were added until 6 days of culture, when nonadherent cells corresponding to the DC-enriched fraction were harvested and washed. Differentiation into mature DCs was confirmed using FACS.

Preparation of cRNA probes

A human IL-23 p19 cDNA fragment containing residues 419–746 (GenBank accession no. AF301620) (4) was amplified using the PCR. The synthesized sense and antisense PCR primers for p19 were 5'–TGGAAAG GATCCCAACAGGGTCTGA-3' and 5'–TGATGTCATCTTCTGGACGT CTGTC–3' (Kurabo) (24). The PCR products were fractionated on agarose gels and then cloned into the pGEM-Easy vector (Promega). Human IL-12 p40 and human G3PDH cDNAs were cloned as described (25).

cRNA probes for human IL-23 p19, IL-12 p40, and G3PDH were synthesized using [α-32P]UTP (ICN Biomedicals) and T7 RNA polymerase (Promega).

Quantitation of IL-23 p19, IL-12 p40, and G3PDH mRNA levels by Northern blotting

We measured cytokine mRNA expression after stimulating THP-1 cells and immature MD-DCs with 1 μg/ml LPS. Total RNA was extracted using a modified acid guanidium thiocyanate-phenol-chloroform method. Northern blots proceeded as described (25). Aliquots of total RNA resolved by electrophoresis were transferred onto nylon membranes and hybridized to human IL-23 p19 or IL-12 p40 cRNA probes at 60°C overnight. Signals for p19 or p40 mRNA were visualized by autoradiography using x-ray film. The probes were then stripped, and the blots were rehybridized with a control human G3PDH cRNA probe.

Measurement of Rac activity

We assayed Rac activity using a kit (Rac Activation Assay Kit; Upstate Biotechnology) according to the manufacturer’s instructions.

Adenovirus construction and infection

We constructed a recombinant replication-deficient adenovirus harboring the genes for dominant-negative Rac1 (N17Rac1) and enhanced GFP (EGFP) (26). Briefly, an N17Rac1 fragment excised from Rac 1 cDNA (dominant-negative) in pUSEamp (Upstate Biotechnology) by digestion with EcoRI and Xhol was inserted into the Swal site of the cosmid vector pAsCAwt, and an adenovirus harboring N17Rac1 (Ad.N17Rac1) was constructed using an adenovirus expression vector kit (Takara Bio). An adenovirus containing EGFP was constructed as described above, except that a fragment was excised from the cDNA in the pEGFP vector (Clontech) by digestion with XbaI. On the day of viral infection, the medium was removed, and cells cultured in 6-well plates were washed once with RPMI 1640. The cells were incubated with adenovirus in 100 μl of medium without serum for 1 h under a humidified 5% CO2 atmosphere at 37°C before complete medium was added. Twenty-four hours later, fresh complete medium was added, and the cells were incubated for a further 24 h before stimulation with LPS.

Western blotting

Western blotting proceeded as described (27). Briefly, cell lysates were electrotransferred and immunoblotted against primary Abs, and then specific reactive proteins were detected using ECL.

Quantitation of IL-12 p70 protein

The concentration of IL-12 p70 (heterodimeric form) in the supernatants was determined using ELISA kits (Quantikine; R&D Systems) according to the manufacturer’s instructions. The assay detected >5 pg/ml IL-12 p70.

Small interference RNA (siRNA) for Rac1 and NF-κB p65

We assessed the effect of inducing RNA interference on Rac1 and NF-κB p65 using commercially available reagents (Silencer Pre-designed siRNA human Rac1 from Ambion and the SignalSilence NF-κB p65 siRNA Kit from Cell Signaling Technology) according to the manufacturer’s instructions. Control siRNA is an siRNA sequence that will not cause the specific degradation of any cellular message. Forty-eight hours after siRNA transfection, we determined Rac1 and NF-κB p65 contents in whole cell lysates by Western blotting using Rac1 (Upstate Biotechnology) and NF-κB p65 Ab (Cell Signaling Technology) to confirm the silencing of these protein.

Transfection of plasmids and luciferase assay

The mammalian expression vector, pGal4-p651-551 was a gift from Dr. Takashi Okamoto (Nagoya City University, Nagoya, Japan). Plasmids pFR-Luc (pGal4-Luc) and pNF-κB-Luc containing five tandem repeats of Gal4-binding sites and NF-κB-binding sites, respectively, were obtained from Stratagene. Cells were transiently transfected using FuGene 6 transfection reagent (Roche) according to the manufacturer’s instructions. All transfections included 2 μg of pGal4-Luc or 1 μg of pGal4-Luc and 50 ng of pGal4-p65 together with plasmid DNA consisting of expression vectors or vector plasmid alone. After transfection, cells were incubated in RPMI 1640 supplemented with 1% FBS for 36 h at 37°C before stimulating with 10 ng/ml LPS for 8 h at 37°C. Luciferase activities were assayed in cell lysates as described in the manufacturer’s protocol (Luciferase Assay System; Promega) normalized by protein concentration and are expressed as light units/μg of protein.

DNA-binding activity of NF-κB

Nuclear extracts were prepared from THP-1 cells using a kit (Nuclear Extract Kit; Active Motif) according to the manufacturer’s instructions.

The DNA-binding capacity of NF-κB was determined using the TransAM NF-κB (Active Motif) as described in the manufacturer’s protocol. Oligonucleotides with the NF-κB consensus site (5’–GGGACCTTCC–3′), to which the active form of NF-κB in the nuclear extracts specifically binds, were immobilized on the assay plates. The activated NF-κB

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p65 and p50 bound to the oligonucleotide was detected using Abs directed against NF-kB p65 and p50, respectively.

**Statistical analysis**

All values are expressed as means ± SEM of the indicated numbers of experiments. Data were compared by Student’s t test with the Bonferroni correction for multiple comparisons. p < 0.05/ (where m is the number of comparisons) was considered statistically significant in the Bonferroni method.

**Results**

**Induction of IL-23 p19 mRNA expression by LPS stimulation in a human macrophage cell line**

Phorbol esters can induce human monocytic THP-1 cells to differentiate into macrophages (20). We reported that 1 μg/ml LPS induces IL-12 p40 mRNA expression in PMA-treated THP-1 cells (28). Here, we examined whether IL-23 p19 mRNA expression is induced by the stimulation of LPS in PMA-treated THP-1 cells. Fig. 1A shows that 1 μg/ml LPS time-dependently increased IL-23 p19 mRNA levels, reaching a maximal response after 8 h in THP-1 cells. The dose-related response of IL-23 p19 mRNA expression to LPS was studied after 8 h of LPS stimulation. Fig. 1B shows that the expression of IL-23 p19 mRNA was dose-dependently up-regulated. Thus, we stimulated cells with 1 μg/ml LPS for 8 h to induce IL-23 p19 mRNA expression in subsequent experiments.

**Rac activation induced by LPS, and N17Rac1 gene transfer by infected adenovirus in human macrophages**

We determined the role of Rac in LPS signal transduction by pull-down assays using PAK-1 agarose to detect Rac-GTP (activated form). LPS maximally activated Rac at 10 min in PMA-treated THP-1 cells and then returned to near basal levels at 30 min thereafter (Fig. 1C).

To evaluate the role of Rac1 in LPS-induced cytokine expression and signal transduction, we infected PMA-treated THP-1 cells for 48 h with a recombinant replication-deficient adenovirus that contained the genes for dominant-negative Rac1 (Ad.N17Rac1). Fig. 2A shows that overexpression of N17Rac1 could be readily ascertained following infection with Ad.N17Rac1 but not uninfected cells or in cells infected with a control adenovirus (Ad.EGFP). Thus, we infected cells with Ad.N17Rac1 for 48 h to induce N17Rac1 overexpression in subsequent experiments.

**Inhibition of Rac1 enhances IL-23 p19 mRNA expression, but minimally affects IL-12 p40 mRNA expression and p70 protein production in human macrophages and DCs**

To examine the role of Rac1 in LPS-induced IL-23 p19 mRNA expression, we Northern blotted total RNA from uninfected, Ad.EGFP-infected, and Ad.N17Rac1-infected THP-1 cells after LPS stimulation. Fig. 2B shows that N17Rac1 overexpression induced by Ad.N17Rac1 infection, but not that of EGFP induced by control Ad.EGFP infection, enhanced IL-23 p19 mRNA expression induced by LPS ~2-fold. Furthermore, in human MD-DCs, infection with Ad.N17Rac1 augmented LPS-induced IL-23 p19
mRNA expression compared with control Ad.EGFP infection (Fig. 2, D and E). On the other hand, the expression of IL-12 p40 mRNA after LPS stimulation did not change in uninfected, Ad.EGFP-infected, or Ad.N17Rac1-infected THP-1 cells (Fig. 2C). The infection of human MD-DCs with Ad.N17Rac1 similarly resulted in an insignificant effect on LPS-induced IL-12 p40 mRNA expression compared with control Ad.EGFP infection (Fig. 2, D and E). Consistent with the mRNA data, infection with Ad.N17Rac1 did not affect LPS-induced IL-12 p70 protein production (Fig. 2F).

To further confirm the role of Rac1 in LPS-induced IL-23 p19 mRNA expression, we performed experiments using Rac1 siRNA. The transfection of THP-1 cells with Rac1 siRNA attenuated the Rac1 content (by 52%) while augmented LPS-induced IL-23 p19 mRNA expression (135%) compared with control siRNA transfection (Fig. 3, A and B). On the other hand, Rac1 siRNA had an insignificant effect on LPS-induced IL-12 p40 mRNA expression compared with control siRNA (Fig. 3C). These results indicate that Rac1 negatively regulates LPS-induced IL-23 p19 mRNA expression but minimally contributes to IL-12 p40 mRNA expression and p70 protein production in human macrophages and DCs.

Inhibition of either p38 MAPK or JNK enhances IL-23 p19 mRNA expression, but inhibition of Rac1 does not affect either JNK or p38 phosphorylation

To further understand the mechanism of LPS-induced IL-23 p19 mRNA expression, we examined the roles of p38 MAPK and JNK using the respective selective inhibitors, SB203580 and SP600125. Fig. 4A shows that SB203580 enhanced IL-23 p19 mRNA expression from human macrophages stimulated with LPS. Similarly, SP600125 augmented LPS-induced IL-23 p19 mRNA expression (Fig. 4B). Neither SB203580 nor SP600125 alone induced IL-23 p19 mRNA expression (Fig. 4, A and B). Although wortmannin, a specific inhibitor of PI3K activity, augmented LPS-induced IL-12 p40 mRNA expression as previously reported (Fig. 4C and Ref. 29), this compound had a small enhancement of IL-23 p19 mRNA expression induced by LPS (Fig. 4D). These results suggest that both the p38 MAP kinase and JNK pathways negatively regulate LPS-induced IL-23 p19 mRNA expression.

We evaluated the role of Rac1 in LPS-induced p38 MAPK and JNK activities. We reported that the LPS-induced activa-

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Effect of Rac1 siRNA on LPS-induced IL-23 p19 and IL-12 p40 mRNA expression. A, Western blots of THP-1 cells without (lane 1) and with 100 nM control siRNA (lane 2) or 100 nM Rac1 siRNA (lane 3) transfection for 48 h. Nitrocellulose filters were probed with anti-Rac1 and anti-p42 MAPK Abs to confirm equal protein loading. B and C, PMA-treated THP-1 cells were transfected with control siRNA or with Rac1 siRNA for 48 h before stimulation with LPS (1 μg/ml). Eight hours (for IL-23 p19) or 24 h (for IL-12 p40) later, total RNA was extracted and expression of IL-23 p19 (B) or IL-12 p40 mRNA (C) was evaluated by Northern blotting as described in the legend to Fig. 1.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Role of p38 MAPK and JNK in LPS-induced IL-23 p19 mRNA expression and Rac1 signaling. A–D, PMA-treated THP-1 cells were cultured with 0.1% DMSO (control vehicle), SB203580 (specific inhibitor of p38 MAPK), or SP600125 (specific inhibitor of JNK) or wortmannin (specific inhibitor of PI3K) for 30 min before stimulation with LPS (1 μg/ml). Eight hours (for IL-23 p19) or 24 h (for IL-12 p40) later, total RNA was extracted, and expression of IL-23 p19 (A, B, D) or IL-12 p40 mRNA (C) was evaluated by Northern blotting as described in the legend to Fig. 1. E and F, PMA-treated THP-1 cells were infected or not with Ad.EGFP or Ad.N17Rac1 for 48 h before stimulation with LPS (1 μg/ml). After 30 min, the activities of p38 MAPK and JNK in whole cell lysates were determined by Western blotting using Abs specific for phosphorylated, activated forms of p38 MAPK (E, top) and JNK (F, top). Corresponding bottom panels are Western blots using Abs to total p38 MAPK (E, bottom) and JNK (F, bottom), indicating amounts of precipitated enzymes.

Inhibition of Rac1 enhances NF-κB-dependent gene expression independently of NF-κB DNA-binding and IκB-α degradation

The NF-κB family of transcription factors plays an important role in LPS-mediated responses (9). Thus, we analyzed NF-κB-dependent gene expression by monitoring the activity of a transfected luciferase reporter gene (5× NF-κB-Luc). Stimulation of THP-1 cells with LPS resulted in a 2- to 3-fold increase in reporter gene activity (data not shown). To evaluate the role of Rac1 in LPS signaling that leads to NF-κB activation, we expressed the NF-κB reporter in uninfected, Ad.EGFP-infected, and Ad.N17Rac1-infected THP-1 cells. Fig. 5A shows that the overexpression of N17Rac1 induced by Ad.N17Rac1 infection significantly enhanced NF-κB luciferase activity with LPS stimulation, whereas EGFP overexpression induced by control Ad.EGFP infection did not affect NF-κB activity. These data indicate that Rac1 activation leads to the inhibition of NF-κB activity in LPS signal transduction.
Inhibition of Rac1 enhances the transcriptional activity of NF-κB p65 but does not affect the phosphorylation of either Ser276 or Ser536

The regulation of NF-κB activation depends not only on IκB phosphorylation but also on the inducible trans activation activity and phosphorylation of p65. We investigated whether LPS-induced signaling through the Rac1 cascade directly leads to transcriptional activation from a Gal4-dependent promoter driven by a chimeric Gal4-p65 protein in THP-1 cells. The stimulation of THP-1 cells with LPS significantly enhanced the Gal4-dependent luciferase activity induced by Gal4-p65 (192 ± 12.2%, n = 6, p < 0.05). The overexpression of dominant-negative Rac1 by Ad.N17Rac1 infection significantly (p < 0.05) up-regulated LPS-induced NF-κB p65 trans activation compared with control Ad.EGFP infection (Fig. 6A). We also examined the effect of dominant-negative Rac1 on the phosphorylation of NF-κB p65. In THP-1 cells, stimulation with LPS caused the phosphorylation of p65 Ser276 and Ser536 (Fig. 6B), but not of Ser529 (data not shown). The phosphorylation of Ser276 or Ser536 induced by LPS in THP-1 cells infected with N17Rac1 adenovirus was not altered compared with cells infected with control EGFP adenovirus (Fig. 6C). These results suggest that the negative effect of Rac1 on NF-κB-dependent gene expression is due to a direct effect on NF-κB p65 trans activation but is independent of p65 Ser276 and Ser536 phosphorylation.

To further examine the effect of LPS-mediated Rac1 signaling on NF-κB activation, we evaluated the effect of Rac1 on NF-κB DNA-binding and IκB-α degradation in PMA-treated THP-1 cells. Fig. 5, B and C, shows that LPS enhanced the DNA-binding capacity of NF-κB p65 and p50, whereas the dominant-negative form of Rac1 (N17Rac1) did not influence the DNA-binding capacity of either p65 (Fig. 5B) or p50 (Fig. 5C) enhanced by LPS. On the other hand, LPS evoked IκB-α degradation, which reached a maximum at 15 min and then returned to near basal levels at 120 min after adding LPS (Fig. 5D). However, the expression of dominant-negative Rac1 did not alter the time course of IκB-α degradation and resynthesis by LPS compared with the expression of control EGFP (Fig. 5E). These results suggested that Rac1 negatively controls the NF-κB-dependent, IκB-independent gene expression triggered by LPS.

FIGURE 5. Effect of dominant-negative Rac1 (N17Rac1) on LPS-induced NF-κB gene expression, IκB degradation, and NF-κB DNA-binding. A–C, PMA-treated THP-1 cells were infected or not with Ad.EGFP or Ad.N17Rac1 for 48 h. A, Cells were transfected with pNFKB-Luc for 36 h before stimulating with LPS. Eight hours later, cells were harvested and luciferase activities were evaluated. The results were expressed as a percentage of the value obtained for control. Data represent means ± SEM of triplicate determinants. *, p < 0.05 compared with Ad.EGFP-infected THP-1 cells stimulated by LPS. Results of two repeated experiments were similar. B and C, After stimulation for 30 min, DNA-binding capacity of NF-κB p65 (B) or p50 (C) was assayed in nuclear extracts. Values represent means ± SEM of four experiments. D, Cells were stimulated with LPS (1 μg/ml) for the indicated periods. IκB-α degradation and phosphorylation was determined in whole cell lysates by Western blotting using Abs specific for total (top) and phosphorylated IκB-α (bottom), respectively. E, Cells were infected with adenovirus as described above and stimulated with LPS for indicated periods; then IκB-α degradation and phosphorylation were determined by Western blotting.

FIGURE 6. Effect of dominant-negative Rac1 (N17Rac1) on LPS-induced transcriptional activity of NF-κB p65 and phosphorylation of Ser276 and Ser536 of p65. A, PMA-treated THP-1 cells were infected with Ad.EGFP or Ad.N17Rac1 for 48 h and then transfected with pGal4-p65 and pGal4-Luc for 36 h and stimulated with LPS (1 μg/ml). Eight hours later, cells were harvested and luciferase activities were assayed. The results were expressed as a percentage of the value obtained for control. Data represent means ± SEM of triplicate determinations. *, p < 0.05 compared with Ad.EGFP-infected THP-1 cells stimulated by LPS. Two repeated experiments yielded similar results. B, Cells were stimulated with LPS (1 μg/ml) for indicated periods. Phosphorylation of NF-κB p65 was determined in whole cell lysates by Western blotting using Abs specific for phosphorylated forms of Ser276 (top) and Ser536 (middle). Corresponding bottom panels are Western blots using Abs to total p65, indicating amounts of precipitated enzymes. C, Cells were infected with adenovirus as described above and stimulated with LPS for 60 min; then NF-κB p65 phosphorylation was determined by Western blotting.
RNA interference of NF-κB p65 suppresses IL-23 p19 mRNA expression but minimally affects IL-12 p40 mRNA expression in human macrophages and DCs

We confirmed the role of the NF-κB p65 subunit in LPS-induced IL-23 p19 mRNA expression using NF-κB p65 siRNA. The transfection of PMA-treated THP-1 cells with NF-κB p65 siRNA attenuated the NF-κB p65 content by 72% compared with control siRNA transfection (Fig. 7A). We examined the susceptibility to LPS-induced IL-23 p19 and IL-12 p40 expression under these conditions. Fig. 7, B and C, shows that NF-κB p65 siRNA reduced the expression of IL-23 p19 mRNA but not that of IL-12 p40 induced by LPS. Densitometry showed that NF-κB p65 siRNA significantly (p < 0.05) inhibited LPS-induced IL-23 p19 mRNA expression compared with control siRNA (Fig. 7D). In human MD-DCs, transfection with NF-κB p65 siRNA suppressed the expression of IL-23 p19 mRNA induced by LPS but not that of IL-12 p40 mRNA as in PMA-treated THP-1 cells (Fig. 7, E and F). These data indicated that regulation of the expression of LPS-induced IL-23 p19, but not of IL-12 p40, is dependent on the NF-κB p65 subunit in human macrophages and DCs.

Discussion

One key finding of the present study was that Rac1 plays a negative role in expression of the IL-23 p19 subunit, but not of the p40 subunit, in human macrophages and DCs stimulated by LPS. Another was that Rac1 negatively regulates LPS-induced NF-κB gene expression through a p65 trans activation-dependent, IκB-independent pathway, resulting in the differential regulation of IL-23 p19 and p40 expression by Rac1. We are the first to report that Rac1 affects IL-23 expression induced by LPS and negatively controls the transcriptional activity of NF-κB p65 in LPS signal transduction.

Although others have described the early signaling events underlying the regulation of IL-12 p40 expression (30), those of IL-23 p19 expression in human APC are not well understood. We previously reported that p38 MAPK positively and JNK negatively regulates LPS-induced IL-12 p40 expression in PMA-treated THP-1 cells (28). The results of the present study demonstrated that blocking the p38 MAPK and JNK signaling pathways with SB203580 and SP600125, respectively, augmented the induction of p19 mRNA expression by LPS (Fig. 3, A and B), indicating that negative signaling pathways for p19 expression are mediated by both p38 MAPK and JNK. In contrast, we consider that IκB-dependent NF-κB signaling, which is important for LPS-mediated responses, positively regulates LPS-induced p19 and p40 expression, given that BAY11-7082, a specific inhibitor of IκB-α phosphorylation, inhibited p19 and p40 mRNA expression stimulated by LPS (data not shown).

The Rho family member, GTPase Rac1, is an important regulator of cytoskeletal organization, but recent reports have also implicated Rac1 in LPS-induced cytokine expression. The gene expression of TNF-α (31) and IL-1 (32) is regulated by Rac1 in mouse macrophages. The present study discovered a Rac1-mediated negative feedback mechanism of LPS-induced IL-23 p19 expression in human macrophages and DCs, because both the overexpression of dominant-negative Rac1 and the transfection of Rac1 siRNA potently enhanced the induction of p19 mRNA expression by LPS (Figs. 2 and 3). By contrast, neither dominant-negative Rac1 nor Rac1 siRNA influenced LPS-induced p40 expression (Figs. 2 and 3), indicating that distinct signaling mechanisms exist between IL-23 p19 and p40 expression.

How Rac1 functions in LPS signal transduction might be related to the finding that both the p38 MAPK and JNK pathways are independent of the Rac1 pathway in LPS signaling, since the inhibition of Rac1 activity did not alter either p38 or JNK activity stimulated by LPS (Fig. 4). On the other hand, the overexpression of dominant-negative Rac1 significantly enhanced LPS-induced NF-κB gene expression without affecting NF-κB DNA binding or IκB-α degradation (Fig. 5). In addition, luciferase assays using pGal4-p65 and pGal4-luc showed that the inhibition of Rac1 activity significantly augmented NF-κB p65 transcriptional activity (Fig. 6A). Therefore, we believe that the LPS-dependent activation of Rac1 negatively controls NF-κB gene expression through the p65 trans activation-dependent, IκB-dependent pathway in human macrophages. TLR2-dependent activation of the Rac1-PI3K-Akt pathway positively regulates the transactivational activity of NF-κB p65 in human monocyte THP-1 cells (19). On the contrary, the present study discovered that Rac1 negatively regulates the transactivational activity of p65 in the TLR4-dependent pathway. Guha and Mackman (33) reported that TLR4-dependent activation of the PI3K-Akt pathway also negatively regulates the transactivational activity of p65 in THP-1 cells. We consider that
the Rac1 and PI3K pathways differentially regulate TLR4-dependent p65 trans activation, because the inhibition of Rac1 activity did not change LPS-induced IL-12 p40 mRNA expression (Figs. 2C and 3C), whereas wortmannin, a selective inhibitor of PI3K, enhanced LPS-induced p40 expression (Fig. 4C). Several studies support the notion that p65 phosphorylation regulates the transcriptional competence of NF-κB (12–14). We showed that the LPS-induced p65 phosphorylation of Ser276 or Ser536 was not affected by the inhibition of Rac1 activity using dominant-negative Rac1 (Fig. 6C). Hence, whether negative regulation of Rac1 in LPS-induced p65 trans activation is independent of phosphorylation of p65 or associated with other phosphorylation site remains obscure and requires further investigation.

Rac1, which affects NF-κB activation, regulated only the expression of p19, whereas NF-κB affected both p19 and p40 expression. NF-κB exists as various homodimeric and heterodimeric complexes, which bind the DNA-regulatory element of target genes in the nucleus. Our study demonstrated that a decrease in cellular amounts of NF-κB p65 caused by p65 siRNA significantly inhibited p19 expression but did not change p40 expression stimulated by LPS (Fig. 7). These findings indicated that a complex containing p65 controls LPS-induced IL-23 p19 mRNA expression.

Because other studies have shown that the p50-cRel heterodimer plays an essential role in LPS-induced p40 expression in macrophages (34) and DCs (35), we believe that NF-κB p65 minimally contributes to IL-12 p40 mRNA expression in human macrophages and DCs. Therefore, Rac1-dependent p65 trans activation controls LPS-induced IL-23 p19 expression but not p40 expression, which causes the differential regulation of IL-23 p19 and p40 expression induced by Rac1.

Recent reports show that IL-23 is an essential factor required for the expansion of a pathogenetic CD4+ T cell population, which is characterized by the production of IL-17, IL-17F, IL-6, and TNF-α (36). Studies using IL-23 p19, IL-12 p40, and p35 knockout mice have established that IL-23 p19 and IL-12 p40, but not IL-12 p35, are essential for the development of T cell-mediated diseases, such as experimental autoimmune encephalomyelitis (37) and collagen-induced arthritis (38). Thus, IL-23, but not IL-12, is essential for the development of autoimmune conditions (6). We demonstrated here that the inhibition of Rac1 activity significantly up-regulated LPS-induced IL-23 p19 expression without affecting IL-12 p70 production (Fig. 2), suggesting that regulation of the Rac1-NF-κB pathway serves as a promising target for the development of new treatment modalities for autoimmune disorders caused by IL-23-dependent CD4+ T cells.

In conclusion, we provided the first evidence of a Rac1-mediated negative feedback mechanism of LPS-induced IL-23 p19 expression by human macrophages and DCs through NF-κB p65 trans activation. In addition, we showed that NF-κB p65 regulates the expression of LPS-induced IL-23 p19 but not of p40, which causes a difference in the Rac1 control of IL-23 p19 and p40 expression. Identifying new targets within the TLR signaling cascades has important implications for the development of new therapeutic strategies aimed at inhibiting NF-κB transcriptions in human disease states involving dysregulation of the immune response, such as autoimmune disorders.

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Disclosures

The authors have no financial conflict of interest.
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