Enhanced IL-10 Production by TLR4- and TLR2-Primed Dendritic Cells upon TLR Restimulation

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Enhanced IL-10 Production by TLR4- and TLR2-Primed Dendritic Cells upon TLR Restimulation

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LPS tolerance has been investigated extensively in monocytes/macrophages. However, the LPS restimulation studies are not well documented in dendritic cells (DCs). In the present study, we investigated influences of TLR restimulation using murine bone marrow-derived DCs. Purified bone marrow-derived DCs (>98% CD11c+ B220−) were stimulated with TLR4 and TLR2 ligands for 24 h and then cultured with medium alone for 48 h as a resting interval (TLR4,2-primed DCs). The TLR4-MD2 expression was markedly reduced immediately after the TLR stimulation, but was restored following the resting interval. The TLR4,2-primed DCs exhibited significantly enhanced IL-10 production, but markedly diminished IL-12p40 production upon TLR4 restimulation compared with naïve (unprimed) DCs. TLR4-mediated activation of p38 MAPK was markedly suppressed, whereas that of ERK1/2 was enhanced in the TLR4,2-primed DCs compared with naïve DCs. Blocking the activation of ERK1/2 with U0126 reduced the enhanced IL-10 production by the TLR4,2-primed DCs upon the TLR4 restimulation. The U0126 showed no significant effects on the IL-12p40 production. Thus, the enhanced ERK1/2 activation appears to be, at least in part, responsible for the enhanced IL-10 production in the TLR4,2-primed DCs. In addition, TNFR-associated factor 3 expression was significantly up-regulated in the TLR4,2-primed DCs compared with that in naïve DCs. We demonstrated in this study that DCs primed with TLR4 and TLR2 ligands and rested for 48 h showed enhanced IL-10 production upon TLR4 restimulation. The enhanced IL-10 production by the TLR4,2-primed DCs may be attributed to the altered balance of intracellular signaling pathways via p38 MAPK, ERK1/2, and TNFR-associated factor 3 upon TLR restimulation. The Journal of Immunology, 2007, 178: 6173–6180.

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Although LPS restimulation studies of monocytes/macrophages have been performed extensively (11–14), those of DCs are not well documented. We performed the LPS restimulation study using murine bone marrow-derived DCs (BMDCs). We demonstrate in this study that DCs primed with TLR4 and TLR2 ligands show enhanced IL-10 production upon TLR4 restimulation after the resting interval that recovers TLR4 expression. The enhanced IL-10 production by the TLR4,2-primed DCs may be attributable to the altered balance of intracellular signaling pathways via p38MAPK, ERK1/2, and TRAF3 upon TLR restimulation.

Materials and Methods

**Mice**

C57BL/6 (B6) mice were purchased from Japan SLC and were maintained in a specific pathogen-free condition at our animal facility at Hokkaido University. All experiments were approved by regulations of Hokkaido University Animal Care and Use Committee.

**Reagents and Abs**

Murine rGM-CSF was purchased from PeproTech. Standard LPS (sLPS) from *Escherichia coli* (055:B5) was obtained from Sigma-Aldrich. Ultra-pure LPS (upLPS) from *E. coli* (0111:B4) and Pam3CSK4 were purchased from InvivoGen. Phosphothioate-stabilized synthetic CpG oligodeoxynucleotides (ODN) (TCCATGACGTTCCTGATGCT) (25) were purchased from Invitrogen and InvivoGen, respectively. Anti-phospho-p44/p42 MAPK (ERK1/2) (Thr202/Tyr204) Ab, anti-phospho-NF-κB p65 (Ser536) Ab, anti-p38 MAPK Ab, anti-phospho-NF-κB p65 (Ser317) Ab, anti-NF-κB p65 Ab, anti-TRAF3 Ab, and streptavidin PerCP were obtained from BD Pharmingen. Anti-phospho-p44/p42 MAPK (ERK1/2) (Thr202/Tyr204) Ab, anti-p44/p42 MAPK (Thr202/Tyr204) Ab, anti-phospho-p38 MAPK (Thr180/Tyr182) Ab, anti-p38 MAPK Ab, anti-phospho-NF-κB p65 (Ser317) Ab, anti-NF-κB p65 Ab, anti-TRAF3 Ab, and HRP-conjugated anti-rabbit IgG Ab were purchased from Cell Signaling Technology. U0126, a specific inhibitor of MEK1/2, and SB203580, a specific inhibitor of p38 MAPK, were purchased from Enzyme Systems Products, CA.

**Culture medium**

RPMI 1640 liquid medium was purchased from Sigma-Aldrich and supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, and 5% FCS.

**DC culture**

Murine BMDCs were generated, as previously described (31). Bone marrow cells were prepared from femur and tibial bone marrow of 6- to 12-wk-old B6 mice. After lysis of erythrocytes, MHC class II-, CD45R (B220)-, CD4-, and CD8-positive cells were removed using MACS separation columns (Miltenyi Biotec). The cells were cultured in 5% FCS/RPMI 1640 supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, and 5% FCS.

**TLR priming**

TLR priming of DCs was conducted in 5% FCS/RPMI 1640 containing GM-CSF (20 ng/ml). BMDCs (3 × 10⁵/ml) were stimulated with sLPS (1 μg/ml), upLPS (1 μg/ml), and/or Pam3CSK4 (300 ng/ml) for 24 h. The cells were washed extensively at least three times and cultured in the absence of TLR ligands for 48 h as a resting interval. The cells were then extensively washed and used as TLR-primed DCs. In some experiments, the TLR-primed DCs were used before the resting interval. As a naive control, DCs were cultured in the absence of TLR ligands for 24 or 72 h.

**Measurement of IL-10 and IL-12**

TLR-primed or naive DCs (2 × 10⁶/ml) were stimulated with sLPS (1 μg/ml), upLPS (1 μg/ml), CpG ODN (1 μg/ml), or Pam3CSK4 (300 ng/ml) in 5% FCS/RPMI 1640 for 24 h, and the culture supernatants were subjected to quantification of the protein level of IL-10 and IL-12p40 by ELISA using OptEIA Set (BD Pharmingen). The dose of each TLR ligand was determined on the basis of its maximum effect on the cytokine productions (data not shown).

In some experiments, cells (2 × 10⁶/ml) were pretreated with U0126 (10 μM), SB203580 (30 μM), or vehicle alone (0.1% DMSO) for 1 h and then treated with upLPS (1 μg/ml) for 24 h in the presence of the inhibitor or vehicle. The culture supernatants were subjected to quantification of the cytokine levels.

**Flow cytometry**

Cell staining with FITC-, PE-, or biotin-conjugated Ab, and streptavidin-PerCP and flow cytometric analysis were performed on EPICS XL (Beckman Coulter), as described in a previous study (32).

**Immunoblotting**

TLR-primed or naive DCs were incubated in 5% FCS/RPMI 1640 for 1 h and then stimulated with upLPS (1 μg/ml) for the indicated time period. Reactions were halted by rapidly cooling on ice, and these DCs were washed by ice-cold PBS. The whole cell lysates were prepared using cell lysis buffer (Cell Signaling Technology). The cell lysates were separated by SDS-PAGE, and then blotted onto a nitrocellulose membrane using iBlot Dry Blotting System (Invitrogen Life Technologies). The membrane was probed with primary Ab, and developed with HRP-conjugated secondary Ab by ECL.

**Statistical analysis**

Student’s t test was used to analyze data for significant differences. Values of p < 0.05 were regarded as significant.

**Results**

**Cell surface expressions of TLR4-MD2 complex and maturation markers on naive and TLR-primed DCs**

In the present study, we performed the LPS restimulation study using purified murine BMDCs (>98% CD11c⁺). The BMDCs were positive for CD11b and negative for CD8 and B220 (data not shown), a pattern typical of conventional myeloid DCs. For the LPS restimulation study, we used two types of LPS, sLPS and upLPS. The sLPS are generally contaminated with other bacterial components such as lipoproteins that activate TLR2-mediated signaling (33), whereas upLPS activates only TLR4-mediated pathway (34). As sLPS had been generally and widely used and might represent the naturally encountering endotoxin, we first examined the DC functions upon LPS restimulation using the sLPS (Figs. 1 and 2).

It has been reported that cell surface expression of TLR4-MD2 complex is markedly reduced by LPS stimulation (15). We first examined the effect of LPS priming on the cell surface expression of TLR4-MD2 on BMDCs. The BMDCs were treated with sLPS for 24 h and then cultured in the absence of sLPS for 48 h as a resting interval. The cell surface expression of TLR4-MD2 was analyzed before and after the resting interval (primed 1 and primed 2, respectively, in Fig. 1A). As a naive control, BMDCs were cultured in the absence of sLPS for 24 or 72 h (naive 1 or naive 2, respectively, in Fig. 1A). Both types of naive DCs expressed substantially lower level of TLR4-MD2. In contrast, the TLR4-MD2 expression was almost completely reduced by sLPS treatment for 24 h (primed 1). After the resting interval, the TLR4-MD2 expression was recovered on the sLPS-primed DCs to the level similar to that on naive DCs (primed 2). Additional sLPS stimulation for 48 h instead of the resting interval never recovered TLR4-MD2 expression on the DCs (data not shown). Thus, the TLR4-MD2 expressing sLPS-primed DCs required the culturing in the absence of sLPS.

We next analyzed the cell surface expression of maturation markers, CD40, CD86, and I-A⁺, on sLPS-primed DCs (Fig. 1B). Moderate levels of CD40, CD86, and I-A⁺ expressions were detected on both types of naive DCs (naive 1 and naive 2 in Fig. 1B).
The expression levels of these molecule were increased by sLPS treatment for 24 h (primed 1). The increased levels of these maturation markers on the sLPS-primed DCs were sustained after the resting interval (primed 2). Because it was possible that the DC purity and population had been changed by the long-term culturing, we evaluated CD11c expression on naive and sLPS-primed DCs after the resting interval. Both naive and sLPS-primed DCs (naive 2 and primed 2 in Fig. 1) exhibited the same CD11c /H11001 B220 /H11002 CD8 /H11002 phenotype as that of DCs before the culture. This finding demonstrated that the DC population was not changed by the culture.

Cytokine productions by LPS-primed DCs upon secondary TLR stimulation

TLR4-MD2 expression was severely reduced on sLPS-primed DCs (primed 1), but recovered after the resting interval (primed 2) (Fig. 1A). We then compared the capability of cytokine production upon sLPS restimulation between primed 1 DCs and primed 2 DCs. IL-10 production by naive DCs (cultured for 24 and 72 h in the absence of sLPS; naive 1 and naive 2, respectively) was significantly increased by sLPS treatment (Fig. 2A, left). In sLPS-primed DCs before the resting interval (primed 1), spontaneous IL-10 production was higher than that by naive DCs (naive 1). However, sLPS-induced IL-10 production in primed 1 group fell within the similar level to that by naive 1 DCs. In contrast, sLPS-primed DCs after the resting interval (primed 2) showed considerably high IL-10 production upon sLPS restimulation compared with those in any other groups (Fig. 2A, left).

In contrast, vigorous IL-12p40 production was shown in naive DCs (naive 1 and naive 2) upon sLPS stimulation (Fig. 2B, right). As compared with the naive DCs, sLPS-primed DCs produced considerably low levels of IL-12p40 upon sLPS restimulation irrespective of before (primed 1) or after the resting interval (primed 2) (Fig. 2A, right).

We then evaluated capability of the sLPS-primed DCs to produce IL-10 in response to the other TLR ligands, Pam3CSK (a synthetic lipopeptide) and CpG ODN, after the resting interval (48 h). Pam3CSK4 or CpG ODN binds to TLR2 or TLR9, respectively, and promotes Th1-like immune responses (35, 36). The sLPS-primed DCs were restimulated with Pam3CSK4 or CpG ODN. The sLPS-primed DCs again showed enhanced IL-10 production upon sLPS restimulation compared with naive DCs (culture medium alone for 72 h) (Fig. 2B). In addition, the sLPS-primed DCs vigorously produced IL-10 in response to either Pam3CSK or CpG ODN. Naive DCs produced negligible amounts of IL-10 in response to these two ligands. On the contrary, IL-12p40 production upon Pam3CSK or CpG ODN stimulation was diminished in the sLPS-primed DCs compared with that in naive DCs. Thus, the enhanced IL-10 production and diminished IL-12 production by sLPS-primed DCs appeared to be detected not only with sLPS restimulation, but also the stimulation through TLRs other than TLR4. However, DCs primed with Pam3CSK or CpG ODN showed no enhanced capability to produce IL-10 in response to the secondary TLR stimulation (data not shown).

Role of TLR2-mediated signal in LPS priming of DCs for enhanced IL-10 production

Other bacterial components such as lipoproteins possibly contaminated in sLPS (33) may stimulate TLR2 during the sLPS priming and affect the capability of the sLPS-primed DCs for cytokine productions. To examine whether the TLR2-mediated signals during the sLPS priming influence the subsequent cytokine production, we performed the TLR restimulation study using upLPS and Pam3CSK. BMDCs were primed with upLPS and/or Pam3CSK for 24 h, and restimulated with upLPS after the resting interval (48 h). DCs primed with upLPS alone showed significantly enhanced IL-10 production upon upLPS restimulation compared with naive DCs cultured with medium alone for 72 h (Fig. 3A, left). However,
the IL-10 production by upLPS-primed DCs was lower than that by sLPS-primed DCs upon sLPS restimulation (compare Fig. 3A, left, with Fig. 2A, left). The priming with Pam3CSK4 alone exerted no significant effects on IL-10 production by the DCs in response to upLPS. In contrast, DCs primed with upLPS plus Pam3CSK4 showed the prominent IL-10 production in response to upLPS compared with naive DCs or DCs primed with upLPS or Pam3CSK4 alone (Fig. 3A, left). The enhanced IL-10 production by both the upLPS- and Pam3CSK4-primed DCs upon restimulation with upLPS was comparable to that by sLPS-primed and sLPS-restimulated DCs (Fig. 2A).

IL-12p40 production by DCs primed with upLPS alone or upLPS plus Pam3CSK4, as compared with that by naive DCs, was again significantly reduced upon upLPS stimulation (Fig. 3A, right). The priming with Pam3CSK4 alone also reduced the IL-12p40 production, although the effect was statistically not significant.

We next analyzed cell surface expression of maturation markers (CD40, CD86, and I-A\(^b\)) and TLR4-MD2 on DCs primed with upLPS plus Pam3CSK4 and rested for 48 h (Fig. 3B). CD40, CD86, and I-A\(^b\) expressions were up-regulated on the TLR4,2-primed DCs compared with those on naive DCs, whereas similar levels of TLR4-MD2 were detected on the TLR4,2-primed DCs and naive DCs (Fig. 3B). In contrast, TLR4-MD2 expression was almost negative on the TLR4,2-primed DCs before the resting interval (data not shown). The TLR4,2-primed DCs exhibited CD11c\(^+\) B220\(^-\) CD8\(^+\) phenotype (data not shown). These findings are almost consistent with those in Fig. 1.

**FIGURE 2.** Enhanced IL-10 and diminished IL-12p40 production by sLPS-primed DCs. A, BMDCs were treated with sLPS (1 \(\mu\)g/ml) for 24 h (Primed 1). The sLPS-treated DCs were washed extensively and then cultured without sLPS for 48 h as a resting interval (Primed 2). As a naive control, DCs were cultured with medium alone for 24 or 72 h (Naive 1 or Naive 2). The cells were restimulated with sLPS for 24 h. The culture supernatants were subjected to quantification of the protein levels of IL-10 and IL-12p40 by ELISA. Each column represents the mean \(\pm\) SE of three independent experiments (*, \(p < 0.05\); **, \(p < 0.01\)). B, BMDCs were treated with sLPS (1 \(\mu\)g/ml) for 24 h, extensively washed, and then cultured without sLPS for 48 h (Primed). As a naive control, DCs were cultured without LPS for 78 h (Naive). The cells were restimulated with CpG ODN (CpG), Pam3CSK4 (P3C), sLPS, or medium alone (Med.) for 24 h. The culture supernatants were subjected to quantification of the protein levels of IL-10 and IL-12p40 by ELISA. Each column represents the mean \(\pm\) SE of three independent experiments.

**FIGURE 3.** Effects of TLR4 and TLR2 priming on IL-10 production upon TLR restimulation. BMDCs were primed with upLPS (1 \(\mu\)g/ml) and/or Pam3CSK4 (P3C, 300 ng/ml) for 24 h, extensively washed, and cultured with medium alone for 48 h as a resting interval. As a naive control, DCs were cultured with medium alone for 78 h (Naive). A, The cells were restimulated with upLPS (1 \(\mu\)g/ml) for 24 h, and amounts of IL-10 and IL-12p40 in the supernatant were quantitated, as described in Fig. 2 legend. Each column represents the mean \(\pm\) SE of four independent experiments (*, \(p < 0.05\); ***, \(p < 0.005\)). B, The cell surface expressions of costimulatory molecules, I-A\(^b\), and TLR4-MD2 on DCs primed with upLPS plus P3C (Primed) and naive DCs were analyzed by flow cytometry. Data were representative of at least four independent experiments with the similar results.

**Altered balance of p38 MAPK and ERK signalings in TLR4,2-primed DCs upon TLR restimulation**

It has been reported that p38 MAPK and ERK1/2 play different roles in IL-10 and IL-12p40 productions (37). Then, we examined whether p38 MAPK and ERK1/2 were differentially regulated in naive and TLR4,2-primed DCs. BMDCs were treated with upLPS plus Pam3CSK4 for 24 h and then cultured without TLR ligands for 48 h (TLR4,2-primed DC). As a control, DCs were cultured with medium alone for 78 h (naive DC). The TLR4,2-primed DCs were stimulated with upLPS for indicated time periods, and intracellular protein levels of active form of p38 MAPK and ERK1/2, phospho-p38 MAPK and phospho-ERK1/2, were determined (Fig. 4). Slight or no phosphorylation of p38 MAPK or ERK1/2 was detected within 10 min after upLPS stimulation in either naive or the TLR4,2-primed DCs (data not shown). Marked phosphorylation of p38 MAPK and ERK1/2 was detected at 10 min after upLPS stimulation in naive or the TLR4,2-primed DCs (data not shown). The levels of phospho-p38 MAPK and phospho-ERK1/2 were decreased at 60 min. The upLPS-mediated phosphorylation of p38 MAPK was significantly reduced in the TLR4,2-primed DCs compared with that in naive DCs. On the contrary, the upLPS-mediated phosphorylation of ERK1/2 was significantly enhanced in the TLR4,2-primed DCs compared with naive DCs. Total protein levels of p38 or ERK1/2 were comparable between native DCs and the TLR4,2-primed DCs.
The TLR4,2-primed DCs were restimulated 24 h and then cultured in the absence of TLR ligands for 48 h (Primed). As a naive control, DCs were cultured with medium alone for 78 h (Naive). The cells were restimulated with upLPS (1 μg/ml) for 30 or 60 min, and whole cell lysates were prepared. Levels of phospho-p38 MAPK (p38), phospho-ERK1/2 (pERK1/2), and ERK1/2 in the cell lysates were determined by immunoblotting. Representative immunoblot of three independent experiments is shown (left). The relative intensity of the specific band is shown (right). Each column represents the mean ± SE of three independent experiments (*, p < 0.05; **, p < 0.01). The cells were restimulated with upLPS (1 μg/ml) and Pam3CSK4 (300 ng/ml) for 24 h, extensively washed, and cultured with medium alone for 48 h (Primed). As a naive control, DCs were cultured with medium alone for 78 h (Naive). The cells were restimulated with upLPS (1 μg/ml) for 30 or 60 min, and whole cell lysates were prepared. Levels of phospho-p38 MAPK (p38), phospho-ERK1/2 (pERK1/2), and ERK1/2 in the cell lysates were determined by immunoblotting. Representative immunoblot of three independent experiments is shown (left). The relative intensity of the specific band is shown (right). Each column represents the mean ± SE of three independent experiments (*, p < 0.05; **, p < 0.01).

FIGURE 5. TLR4-induced activation of NF-κB pathway in naive and TLR4,2-primed DCs. BMDCs were primed with upLPS and Pam3CSK4 for 24 h and then cultured in the absence of TLR ligands for 48 h (TLR4,2-primed DC). The TLR4,2-primed DCs were restimulated with upLPS for indicated time periods. Spontaneous phosphorylation of NF-κB p65 was detected in both naive and TLR4,2-primed DCs, but the level of phospho-NF-κB p65 in the TLR4,2-primed DCs was significantly higher than that in naive DCs (Fig. 5). Treatment with upLPS showed negligible effects on phosphorylation of NF-κB p65 in naive and the TLR4,2-primed DCs within 10 min after upLPS stimulation (data not shown). The level of phospho-NF-κB p65 was markedly increased at 30 and 60 min after upLPS stimulation.

p65 in cytoplasm is phosphorylated and subsequently translocated into the nucleus.

We then examined intracellular protein levels of phospho-NF-κB p65. BMDCs were treated with upLPS plus Pam3CSK4 for 24 h and then cultured in the absence of TLR ligands for 48 h (TLR4,2-primed DC). The TLR4,2-primed DCs were restimulated with upLPS for indicated time periods. Spontaneous phosphorylation of NF-κB p65 was detected in both naive and TLR4,2-primed DCs, but the level of phospho-NF-κB p65 in the TLR4,2-primed DCs was significantly higher than that in naive DCs (Fig. 5). Treatment with upLPS showed negligible effects on phosphorylation of NF-κB p65 in naive and the TLR4,2-primed DCs within 10 min after upLPS stimulation (data not shown). The level of phospho-NF-κB p65 was markedly increased at 30 and 60 min after upLPS stimulation.

FIGURE 4. Altered balance of p38 MAPK and ERK1/2 signaling pathways in TLR4,2-primed DCs upon TLR4 restimulation. BMDCs were primed with upLPS (1 μg/ml) and Pam3CSK4 (300 ng/ml) for 24 h, extensively washed, and cultured with medium alone for 48 h (Primed). As a naive control, DCs were cultured with medium alone for 78 h (Naive). The cells were restimulated with upLPS (1 μg/ml) for 30 or 60 min, and whole cell lysates were prepared. Levels of phospho-NF-κB p65 (pp65) and NF-κB (p65) in the cell lysates were determined by immunoblotting. A representative immunoblot of three independent experiments is shown (left). The relative intensity of the specific band is shown (right). Each column represents the mean ± SE of three independent experiments (*, p < 0.05; **, p < 0.01). The levels of phospho-NF-κB p65 in the TLR4,2-primed DCs at these points were higher than those in naive DCs, although the difference was not impressive compared with that in the spontaneous phosphorylation. Total protein levels of NF-κB p65 were comparable between naive and the TLR4,2-primed DCs.

The role of ERK1/2 pathway in enhanced IL-10 production by TLR4,2-primed DCs

As compared with naive DCs, TLR4,2-primed DCs showed enhanced activation of ERK1/2 upon restimulation with upLPS (Fig. 4). By contrast, p38 MAPK activation was considerably reduced. To elucidate roles of ERK1/2 and p38 pathways in the differential balance of IL-10 and IL-12p40 productions in naive and TLR4,2-primed DCs, blocking study was performed using U0126, a specific inhibitor of MEK1/2 that is required for ERK1/2 activation, and SB203580, a specific inhibitor of p38 MAPK, during the TLR restimulation.

BMDCs were primed with upLPS plus Pam3CSK4 for 24 h and then cultured with medium alone for 48 h (TLR4,2-primed DCs). As a control, DCs were cultured with medium alone for 78 h (naive DCs). The TLR4,2-primed or naive DCs were pretreated with U0126, SB203580, or vehicle alone (DMSO) for 1 h and then stimulated with upLPS for 24 h in the presence of each inhibitor. The TLR4,2-primed DCs again showed enhanced or diminished production of IL-10 or IL-12p40, respectively, upon upLPS stimulation compared with that by naive DCs (Fig. 6). U0126 significantly decreased IL-10 production by the TLR4,2-primed DCs was slightly decreased by upLPS stimulation in both naive and the TLR4,2-primed DCs (Fig. 5). As compared with naive DCs, TLR4,2-primed DCs showed enhanced activation of ERK1/2 upon restimulation with upLPS. By contrast, p38 MAPK activation was considerably reduced. To elucidate roles of ERK1/2 and p38 pathways in the differential balance of IL-10 and IL-12p40 productions in naive and TLR4,2-primed DCs, blocking study was performed using U0126, a specific inhibitor of MEK1/2 that is required for ERK1/2 activation, and SB203580, a specific inhibitor of p38 MAPK, during the TLR restimulation.

FIGURE 6. IL-10 and IL-12 production by TLR4,2-primed DC in the presence of ERK1/2 or p38 MAPK inhibitor. BMDCs were primed with upLPS (1 μg/ml) and Pam3CSK4 (300 ng/ml) for 24 h, extensively washed, and cultured with medium alone for 48 h (Primed). As a naive control, DCs were cultured with medium alone for 78 h (Naive). The cells were pretreated with 10 μM U0126 (a MEK1/2 inhibitor) or 30 μM SB203580 (SB, a p38 MAPK inhibitor) for 1 h and then restimulated with upLPS (1 μg/ml) for 24 h in the presence of each inhibitor. Each column represents the mean ± SE of three independent experiments (*, p < 0.05; **, p < 0.01).
TRAF3 was determined by immunoblotting. It is shown in Fig. 7 that substantial intracellular protein levels of TRAF3 were present in both naive or TLR4,2-primed DCs. BMDCs were primed with upLPS plus Pam₃CSK₄. After the resting interval (48 h), intracellular protein levels of TRAF3 were determined by immunoblotting. A representative immunoblot of three independent experiments is shown. The relative intensity of the specific band is shown. Each column represents the mean ± SE of three independent experiments (∗, p < 0.05).

Enhanced TRAF3 expression in TLR4,2-primed DCs

Recently, Hacker et al. (8) have reported that TRAF3 directly associates with MyD88 and TIR domain-containing adaptor-inducing IFN-β after activation of these adaptor molecules and is required for TLR-mediated production of IL-10, but not IL-12p40. We then compared TRAF3 levels in naive and TLR4,2-primed DCs. BMDCs were primed with upLPS plus Pam₃CSK₄. After the resting interval (48 h), intracellular protein levels of TRAF3 were determined by immunoblotting. It is shown in Fig. 7 that substantial levels of TRAF3 are expressed in naive DCs. Of note, the level of TRAF3 was significantly increased in the TLR4,2-primed DCs compared with that in naive DCs.

Discussion

Macrophages/Monocytes and DCs are activated by cell wall products of Gram-positive (38–40) and Gram-negative bacteria (41, 42), spirochetes (43), yeast (39), and mycobacteria (39, 44) via TLR4 and TLR2 in the innate immune system. TLR4 binds to LPS, whereas TLR2 recognizes bacterial lipoproteins and peptidoglycan by forming a heterodimer with TLR1 or TLR6 (45). In the present study, we demonstrated that purified BMDCs primed with TLR4 and TLR2 ligands exhibited enhanced IL-10 and diminished IL-12 production upon secondary TLR4 stimulation after a resting interval.

To date, most TLR restimulation studies of macrophage and DCs have been performed without the resting interval between first and second stimulations. It has been reported that TLR4-MD2 expression on macrophages is severely down-regulated by LPS treatment (15). We also confirmed the considerable down-modulation of TLR4-MD2 on BMDCs by sLPS treatment. However, the TLR4-MD2 expression was recovered to the level comparable to that on naive DCs after culturing with medium alone for 48 h as the resting interval. sLPS-primed DCs acquired the enhanced capability to produce IL-10 after the resting interval. It seems that the enhanced capability is associated with the recovering of TLR4-MD2 expression after sLPS restimulation.

On the contrary, sLPS-primed DCs showed impaired IL-12p40 production in response to sLPS restimulation. It has been reported that the hyporesponsiveness to the secondary LPS stimulation is attributable to the receptor down-regulation and/or the suppressed intracellular signaling (11–15). The impaired IL-12p40 production by sLPS-primed DCs was observed even after the recovering of TLR4-MD2 expression. Thus, the impaired IL-12p40 production appeared to be attributable to the suppressed intracellular signaling rather than the receptor down-modulation. Indeed, the TLR4,2-primed DCs after the recovering of TLR4-MD2 expression showed reduced activation of p38 MAPK, which is responsible for IL-12 synthesis (37), upon TLR4 restimulation.

We used two types of LPS, sLPS and upLPS, for the LPS restimulation study. Contaminated lipoproteins in sLPS may stimulate DCs through TLR2 (33), whereas upLPS activates only TLR4 pathway (34). The priming with upLPS alone significantly increased capability of DCs to produce IL-10 upon upLPS restimulation, whereas ERK1/2 activity in TLR4,2-primed DCs was lower than that of sLPS-primed DCs. This finding suggested that the contaminating lipoproteins also contributed to the enhanced capability of sLPS-primed DCs to produce IL-10. To examine this possibility, function of DCs primed with upLPS plus Pam₃CSK₄ for IL-10 production was evaluated. Upon TLR4 restimulation, the TLR4,2-primed DCs showed the IL-10 production comparable to that of sLPS-primed DCs. This effect of Pam₃CSK₄ was similarly observed at a low concentration (10 ng/ml) (data not shown). It seems that TLR4-mediated signal during the sLPS priming of DCs is also responsible for the enhanced capability of the IL-10 production. In contrast, similar levels of hyporesponsiveness for the IL-12p40 production on TLR4 restimulation were observed in TLR4,2-primed DCs and TLR4-primed DCs. It is likely that the LPS tolerance for IL-12p40 production is fully established by TLR4-mediated signal alone during the DC priming.

Although transcription factors and signal transduction pathways for production of proinflammatory cytokines have been well identified (46), those of anti-inflammatory cytokines such as IL-10 are not well characterized. Consequently, mechanisms underlying LPS tolerance for generation of proinflammatory cytokines have been well documented (11–14), whereas the regulatory mechanisms of IL-10 production upon LPS restimulation remained elusive. In the present study, we focused on the mechanism underlying prominent LPS tolerance for IL-10 production by TLR4,2-primed DCs upon LPS restimulation. MAPK pathways are involved in a variety of cytokine synthesis. It has been reported that ERK1/2 pathway is responsible for IL-10 production (47–49), while showing inhibitory or no effect on IL-12 production (49–51). Thus, we analyzed ERK1/2 activity in the TLR4,2-primed DCs upon restimulation through TLR4. TLR4,2-primed DCs after the resting interval showed enhanced ERK1/2 activation upon TLR4 restimulation compared with that in naive DCs. Blocking the activation of ERK1/2 with U0126 significantly reduced the enhanced IL-10 production by the TLR4,2-primed DCs, while showing no significant effects on the IL-12p40 production. Thus, the enhanced ERK1/2 activation appears to be responsible for the enhanced IL-10 production by TLR4,2-primed DCs. However, the effect of the ERK1/2 inhibition on the IL-10 production was partial, suggesting a possibility that the other pathway is also involved in the enhancement of IL-10 production.

Recently, TRAF3 has been characterized as a key molecule for IL-10 production in response to TLR stimulation (8, 9). Gene targeting study indicates that TRAF3 is essential for TLR-mediated production of IL-10 and type I IFNs. In contrast, TRAF3 is dispensable for activation of TLR6-dependent signal cascades, such as MAPK pathways and subsequent IL-12 production upon TLR stimulation (8). In the present study, the TRAF3 level in TLR4,2-primed DCs was significantly up-regulated compared with that in naive DCs. We considered that TLR-mediated activation of TRAF3 pathway was enhanced in the TLR2,4-primed DCs and involved in the augmented IL-10 after stimulation with upLPS. To...
date, however, downstream transcriptional factors of TRAF3 responsible for the IL-10 production remain unclear.

It has been reported that p38 MAPK promotes not only IL-12, but also IL-10 productions in response to TLR stimulation (52). In the present study, however, TLR4-mediated p38 MAPK activation was significantly reduced in TLR4,2-primed DCs compared with naïve DCs upon restimulation with LPS. Nevertheless, the TLR4,2-primed DCs showed enhanced IL-10 production compared with naïve DCs. In the TLR-primed DCs, highly activated ERK1/2 and TRAF3 pathways that lead selective production of IL-10 appeared to compensate for the reduced activation of p38 MAPK pathway. This altered balance of intracellular signalings in the TLR-primed DCs might result in the enhanced and reduced production of IL-10 and IL-12p40, respectively, upon TLR restimulation.

Blocking p38 MAPK pathway with SB203580 significantly inhibited TLR4-mediated IL-12p40 production by naïve DCs. On the contrary, the blocking of p38 MAPK significantly increased TLR4-mediated IL-12p40 production in the TLR4,2-primed DCs, while decreasing IL-10 production. It has been reported that IL-10 suppresses production of proinflammatory cytokines, including IL-12 (53). Thus, it seemed possible that the increase of IL-12p40 production resulted from the low level of IL-10 production in the SB203580-treated DCs primed with TLR4 and TLR2 ligands and restimulated with LPS. However, addition of exogenous IL-10 (10 ng/ml) in the DC culture showed modest effect on TLR4-mediated IL-12p40 production by either naïve or TLR4,2-primed DCs, and the increase in IL-12p40 production by blocking p38 MAPK was similarly observed in the presence of the excess amount of exogenous IL-10 (data not shown). In addition, it has been demonstrated that functional role of p38 MAPK in the CD40-mediated IL-12p40 production is different between immature and mature (LPS-stimulated) DCs (51, 54). Thus, p38 MAPK may negatively regulate TLR4-mediated IL-12p40 production in the TLR-primed DCs, but not in the naïve DCs.

The differential roles of ERK and p38 MAPK in regulation of IL-10 vs IL-12 secretion by DCs have been reported by several studies using various culture systems (49, 55–57). Consistent with these previous studies, we also demonstrated the differential roles of ERK and p38 MAPK in IL-10 and IL-12 productions by TLR2,4-primed DCs upon TLR stimulation. Because IL-10 vs IL-12 secretion by DCs has important functional implications in skewing a Th1 or Th2 response, the regulation of ERK and p38 MAPK balance in DCs seems to be a target to regulate undesirable immune responses such as allergy and autoimmunity.

It has been demonstrated that c-Fos plays a crucial role for regulation of the IL-10/IL-12p40 balance produced by human monocyte-derived DCs and mouse splenic DCs (55–57). In contrast, sustained ERK signaling phosphorylates and stabilizes c-Fos (58). Thus, it is possible that the enhanced ERK activity in TLR2,4-primed DCs upon LPS stimulation results in the increased phosphorylation of c-Fos. Agrawal et al. (55) showed that Pam3cs, a TLR2 ligand, induced phosphorylation of c-Fos in human monocyte-derived DCs. In the present study, we also analyzed phosphorylation of c-Fos in naïve and TLR4,2-primed DCs upon LPS stimulation. However, we were unable to detect phosphorylated c-Fos in neither naïve DCs nor TLR4,2-primed DCs (data not shown). To date, the role of c-Fos in the enhanced IL-10 production by TLR4,2-primed DCs remains unclear.

In the present study, we used B6 mice, one of the most popular Th1-dominant strains. Thus, it remains to be investigated whether the DC-tolerant state observed in the present study is also seen in a Th2-dominant mouse strain. Strain difference might influence IL-10 vs IL-12 balance in TLR2,4-primed DCs. Recently, it has been reported that splenic DCs prepared from mice at a late phase of polymicrobial sepsis are unable to secrete IL-12, but released high levels of IL-10 in response to CpG ODN or LPS plus CD40L (59). The splenic CD4+CD8- and CD4+CD8+ subpopulations were selectively lost during the sepsis. Perry et al. (60) reported that splenic DCs from malaria-infected mice at late stages exhibited enhanced IL-10 and decreased IL-12 production upon TLR stimulation. The enhanced capability of these ex vivo DCs to produce IL-10 may be attributable to the alternation of DC subpopulations and/or DC function following priming with these bacterial components. However, the precise mechanism remains unclear.

We demonstrated in this study that DCs primed with TLR4 and TLR2 ligands exhibited altered activation state of intracellular signal pathway and enhanced IL-10 and diminished IL-12 production upon TLR4 restimulation after recovering the TLR4-MD2 expression. Our present findings may elucidate the mechanism underlying the enhancement of TLR-mediated IL-10 production by DCs from infected mice.

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Disclosures
The authors have no financial conflict of interest.

References


