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Differential regulation of indoleamine 2,3-dioxygenase by lipopolysaccharide and interferon gamma in murine bone marrow derived dendritic cells

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Abstract **Indoleamine 2,3-dioxygenase (IDO) is a rate-limiting enzyme in the L-tryptophan-kynurenine pathway, which converts an essential amino acid, L-tryptophan, to *N*-formylkynurenine. The expression of IDO increases when inflammation is induced by wounding, infection or tumor growth. Although recent studies have suggested that IDO expression is up-regulated by IFN-γ in various cell types and that the induction of IDO can also be mediated through an IFN-γ-independent mechanism, these mechanisms still remain unknown. In this study, we investigated whether lipopolysaccharide (LPS) induces the expression of IDO through an IFN-γ-mediated signaling pathway or not. IFN-γ-induced expression of IDO expression was inhibited only by JAK inhibitor I. However, LPS-induced expression of IDO was inhibited by LY294002 and SP600125 but not by JAK inhibitor I, SB203580, or U0126. These findings clearly indicate that LPS can induce the IDO expression via an IFN-γ-independent mechanism and PI3 kinase and JNK in the LPS-induced pathway leading to IDO expression.**

Keywords: Indoleamine 2,3-dioxygenase; Dendritic cells; Lipopolysaccharide

1. Introduction

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that mediates the initial and rate-limiting step in the catabolism of tryptophan along the kynurenine pathway [1]. It is well-known that pro-inflammatory mediators such as endotoxin and interferon-γ (IFN-γ) induce the expression of IDO in several tissues. The biological role of IDO in the immune system has been implicated in the inhibition of T cell proliferation by trytophan catabolism, which protects the fetus from maternal responses. Previous studies have shown that human dendritic cells (DCs) expressing significant IDO activity can mediate the inhibition of T cell proliferation through the degradation of tryptophan [2]. The overall immunosuppressive effects of IDO are not entirely well-known, but may depend on the type of IDO-expressing cell [3]. Although the mechanism of IDO induction in vivo is not completely clear, it is widely accepted that interferons, particularly IFN-γ, are essential stimulators for IDO induction [4].

IFN-γ signals through a pair of receptor subunits comprising IFN-γR1 and IFN-γR2. The IFN-γR1 subunit is constitutively associated with Jak1, whereas IFN-γR2 is associated with Jak2 [5]. The binding of IFN-γ to IFN-γ R results in the cross-activation of these Jak protein tyrosine kinases, which then phosphorylate downstream substrates such as Stat1 and Stat2 [5]. According to the above signaling pathway, IFN-γ induces the expression of a number of cellular genes, resulting in induced synthesis of many gene products, including MHC classes [6–8], Fc receptors [9], enzyme activity, *e.g.* IDO [10] and many other proteins of unknown function [11].

It has previously been reported that IDO induction can also be mediated through an IFN-γ-independent mechanism under certain circumstances [12,13]. However, little is known about the signal transduction mechanism responsible for LPS-induced expression of IDO, which occurs through an IFN-γ-independent mechanism. Fujigaki et al. [14] reported that LPS-induced IDO expression is not regulated by an IFN-γ-dependent mechanism, and may be related to the activities of the p38 MAPK pathway and NF-κB.

A variety of signaling pathways in the response to LPS have been well-elucidated; this pathway leads to the activation of NF-κB via Toll-like receptor 4 (TLR4). Although the NF-κB pathway is undoubtedly the key factor in the response to LPS, the role of the MAPK pathways is less certain. Mitogen-activated protein kinases (MAPKs) are involved in perpetuating the inflammatory response, and are activated through well-defined signaling pathways [15]. When MyD88 knockout mice were stimulated with LPS, LPS-induced activation of MAPK was delayed compared with wild-type mice, which suggested that MyD88 is involved, but is not a necessary molecule [16]. Moreover, Mal/Tirap, an adaptor molecule that is homologous to MyD88, has been shown to be involved in the activation of MAPKs by TLR4 [17]. PI3K is a mediator for TLR4-MyD88-mediated JNK activation after LPS stimulation in macrophages [18], although upstream molecules in the JNK pathway in bone

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marrow derived dendritic cells (BMDCs) are unknown. We queried whether JNK may also be involved in LPS-induced expression of IDO, and if so, whether PI3K participates in LPS-induced activation of JNK.

In the present study we show that LPS stimulation induces IDO gene expression in BMDCs and that this induction occurs through PI3K- and JNK-dependent pathways. Finally, we propose that up-regulation of IDO gene expression following LPS stimulation in BMDCs is mediated by an IFN-γ-independent mechanism. These studies provide new insights into the mechanism by which induction of IDO gene expression responds to LPS in BMDCs.

2. Materials and methods

Mice. Male 8-12 weeks-old C57BL/6 (H-2K^b and I-A^b) and BALB/c (H-3K^d and I-A^d) mice were purchased from the Korean Institute of Chemistry Technology (Daejeon, Korea). They were housed in a specific pathogen-free environment within our animal facility for at least one week before use.

2.1. Reagents and Abs

Materials were obtained from the following sources: recombinant mouse (rm) GM-CSF and rmIL-4 were purchased from R&D Systems. Various inhibitors such as InSolutionTM JAK Inhibitor I, LY294002, SP600125, SB203580 and U0126 were obtained from Calbiochem (San Diego, CA); LPS (from Escherichia coli 055:B5) was obtained from Sigma (St. Louis, MO); FITC- or PE-conjugated anti-CD11c (HL3) by flow cytometry, as well as isotype-matched control mAbs, biotinlyated-anti-CD11c (N418) mAbs, were purchased from eBioscience (San Diego, CA); PE-conjugated anti-p-JNK and anti-p-c-Jun were purchased from Santa Cruz (Santa Cruz, CA); Mouse anti-IDO monoclonal Ab and FITC-conjugated Goat anti-Mouse IgG were purchased form Chemicon International (Temecula, CA). anti-phospho-ERK, anti-ERK, anti-phospho-p38, anti-p38, anti-phospho-JNK, anti-JNK, anti-phospho-Akt, anti-Akt and α-tubulin were purchased from Santa Cruz.

2.2. Generation and culture of DCs

DCs were generated from murine BM cells according to the procedure of Inaba et al. [19] with minor modification. Briefly, BM was flushed from the tibiae and femurs of 6-8 weeks male C57BL/6 and depleted of red blood cells with Red Blood cell Lysing buffer (Sigma). The cells were plated in 6-well culture plates (1×10^6cells/ml; 2 ml/well) in RPMI supplemented with 10% heat-inactivated FBS, 100 U/ml penicilline, 100 mg/ml stereptomycin, 20 ng/ml rmGM-CSF and 10 ng/ml rmIL-4 at 37 °C in 5% CO_2. On days 3 and 5 of the culture, floating cells were gently removed and fresh medium was added. On day 6 of the culture, non-adherent cells and loosely adherent proliferating DC aggregates were harvested for analysis or stimulation. On day 7, 80% or more of the non-adherent cells expressed CD11c.

2.3. PI3 Kinase activity

PI3K activity was evaluated by competitive ELISA (Echelon Biosciences). Cells were washed 3× in iced buffer (in mmol/L: 137 NaCl, 20 Tris–HCl, pH 7.4, 1 $CaCl_2$, 1 MgCl, and 0.1 Na_3VO_4), lysed by addition of 1% NP-40 and 1 mmol/L PMSF, and lysates centrifuged for 10 min at 13000 rpm at 4 °C. PI3K pulled down by anti-PI3K p85 antibody (Upstate Biotechnology) and protein A/G beads (Santa Cruz Biotechnology) from 100-μg cell lysates. PI3K activity was assessed using phosphatidylinositol 4,5 bisphosphate as the substrate.

2.4. Quantitative real-time PCR

IDO PCR primers used were as follows: forward 5′-GTACATCACCATGGCGTATG-3′, reverse 5′-CGAGGAAGAAGCCCTTGTC-3′. Quantitative amounts of each gene was standardized against the GAPDH housekeeping gene. Real-time PCR was performed using a BioRad MiniOpticon System (BioRad Laboratories Ltd.) with SYBR green fluorophore. Reactions were performed in a total volume of 20 μl-including 10 μl 2× SYBR Green PCR Master Mix (Applied Biosystems), 1 μl of each primer at 10 μM concentration and 1 μl of the previously reverse-transcribed cDNA template. The protocols used are as follows: denaturation (95 °C for 10 min), amplification repeated 40 times (95 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s, and acquisition temperature for 15 s). For each sample, ddCt (crossing point) values were calculated as the Ct of the target gene minus the Ct of the GAPDH gene. Gene expression was derived according to the equation 2^{-ddCt}; changes in gene expression were expressed as relative to basal.

2.5. Flow cytometry

Cells (1×10^5) were washed in PBS containing 0.1% sodium azide and incubated with 10% (v/v) normal goat serum to block non-specific binding of IgG for 15 min at 4 °C and stained with FITC- or PE-conjugated mAbs CD11c (N418) for 30 min at 4 °C. For Intracellular staining of IDO, p-JNK and p-c-Jun were assayed by flow cytometry as previously reported [20]. The cells (1×10^5) were analyzed using the FACS Calibur (Becton-Dickinson).

2.6. In vivo treatment of JNK inhibitor, SP600125 in mice

JNK inhibitor (SP600125, 30 mg/kg intraperitoneal injection), unlike older non-specific inhibitors, has been shown to be specific for JNK activity at the concentrations used herein [21,22]. The dose was chosen on the basis of previous in vivo studies that showed 30 mg/kg inhibited JNK activity [22]. For analysis of intracellular IDO expression in splenic DCs using flow cytometry, Mice were intraperitoneally injected with SP600125 (30 mg/kg) for 12 h before the administration of 1 mg/kg of LPS. After 18 h from LPS challenge, spleens from mice were taken out. Their spleens were disrupted and the cells were centrifuged at 400×*g* for 5 min and remove red blood cells from mouse splenocytes by treatment of red blood cell lysing buffer (Sigma–Aldrich, St. Louis, MO, USA). And then the cells were harvested and washed twice with phosphate buffered saline (PBS) containing with 2% fetal bovine serum and 0.1% sodium azide.

2.7. Statistical analysis

Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the means ± S.E.M. ANOVA was used to compare experimental groups to control values. While comparisons between multiple groups were done using Tukey's Multiple Comparison test. Statistical significance was determined as P value less than 0.05.

3. Results

3.1. LPS and IFN-γ induces IDO Expression on BMDCs in a dose-dependent fashion

Initially, we performed dose-response studies of LPS- and IFN-γ-induced IDO expression on BMDCs using quantitative real-time PCR and flow cytometry. BMDCs were cultured with LPS (0–800 ng/ml) or IFN-γ (0–400 U/ml) for 24 h. Fig. 1A shows that the level of IDO mRNA expression by LPS or IFN-γ was enhanced in a dose-dependent manner. In particular, the level of IDO mRNA in BMDCs treated with LPS or IFN-γ stimulation was maximal at 200 ng/ml (4.86-fold induction) or 100 U/ml (12.65-fold induction), respectively. Fig. 1B shows that the level of IDO protein production induced by LPS or IFN-γ was enhanced in a dose-dependent manner. The induction of IDO protein by LPS and IFN-γ was maximal at 200 ng/ml (Fig. 1B-d) and 100 U/ml (Fig. 1B-i), respectively. Therefore, we decided to use a suboptimal dose of LPS, as 200 ng/ml, and of IFN-γ, as 100 U/ml, for the later experiments.

3.2. LPS increases IDO expression via an IFN-γ-independent mechanism in BMDCs

Since PI3K is one of the major target molecules in TLR4-mediated signaling in various cell types [23,24] and JAK is

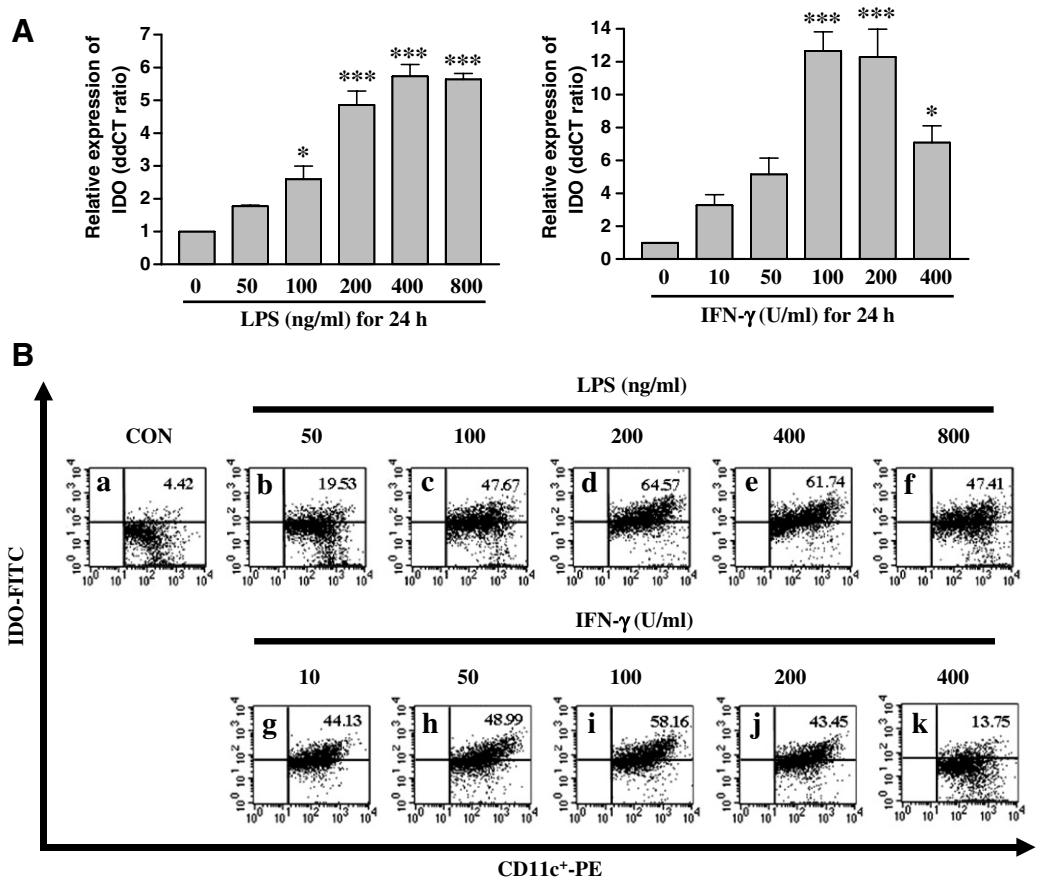

Fig. 1. Effects of LPS and IFN-γ on IDO expression in murine BMDCs. (A) DCs were treated with indicated concentrations of LPS or IFN-γ for 24 h, and then cells were harvested and total RNA was extracted. Quantitative mRNA expression was measured as described in Section 2. (B) DCs were treated with indicated concentrations of LPS and IFN-γ for 24 h, and then cells were harvested and analyzed by flow cytometry. The cells were gated on $CD11c^+$. Values are means ± S.E.M. obtained from at least three separate experiments. The asterisks in panel A indicate significant increases compared to that of non-treated DCs at $P < 0.05$ (*) and 0.001 (***).

constitutively associated with the IFN-γR1 subunit, we first sought to determine whether IDO expression by LPS stimulation is mediated by TLR4 or IFN-γR1 in BMDCs. Accordingly, we used LY294002 as a PI3K inhibitor and JAK inhibitor I as a JAK inhibitor. Fig. 2A shows that LPS-induced IDO mRNA expression was completely inhibited by only LY294002, but not by JAK inhibitor I at the concentration at which IFN-γ-induced IDO gene expression was abolished. In contrast, IFN-γ-induced IDO mRNA expression was inhibited by only JAK inhibitor I but not by LY294002. As shown in Fig. 2B, the protein level of IDO induced by LPS stimulation was also completely inhibited in LPS-treated DCs in the presence of LY294002 (Fig. 2B-e) but not in the presence of JAK inhibitor I (Fig. 2B-h). However, the protein level of IDO induced by IFN-γ stimulation was completely inhibited in IFN-γ-treated DCs in the presence of the JAK inhibitor I (Fig. 2B-i) but not in the presence of LY294002 (Fig. 2B-f). Next, to test whether PI3 kinase is activated by exposure to LPS in murine dendritic cells, we examined the time course of PI3 kinase activity after LPS stimulation. LPS-induced PI3 kinase activation reached a maximum value at 15 min after LPS stimulation, and decreased thereafter (Fig. 2C). One of the downstream targets of PI3 kinase is Akt/protein kinase B [25]. To confirm the effect of LPS on PI3 kinase activity, PI3 kinase activation was evaluated by Western blot analysis of the phosphorylated Akt after LPS exposure in DCs. As shown in Fig. 2D, the phosphorylation of Akt tended to increase with 5 min of exposure to LPS and increased at 30 min of LPS incubation. The phosphorylation of Akt remained close to this level thereafter. In addition, DCs were treated 1 h before LPS stimulation with PI3K inhibitor, LY294002 (10 μM). Incubation with LY294002 resulted in marked inhibition of Akt phosphorylation (Fig. 2E). These data suggest that LPS-induced expression of IDO is regulated by PI3K, and may not be regulated by IFN-γ-mediated JAK signaling.

3.3. JNK is involved in LPS-induced IDO gene expression in BMDCs

To examine the possible role of MAPK in the signal transduction responsible for LPS- or IFN-γ-induced IDO expression, we analyzed the effect of three different MAPK inhibitors; SP600125 as a JNK inhibitor, SB203580 as a p38 MAPK inhibitor, and U0126 as an ERK1/2 inhibitor. The results show that these inhibitors have no inhibitory effect on IFN-γ-induced IDO expression, suggesting that MAPKs are not involved in the induction of IDO expression by IFN-γ (Fig. 3A, *right* and B-f,i,l). However, LPS-induced IDO expression was down-regulated by only SP600125, which

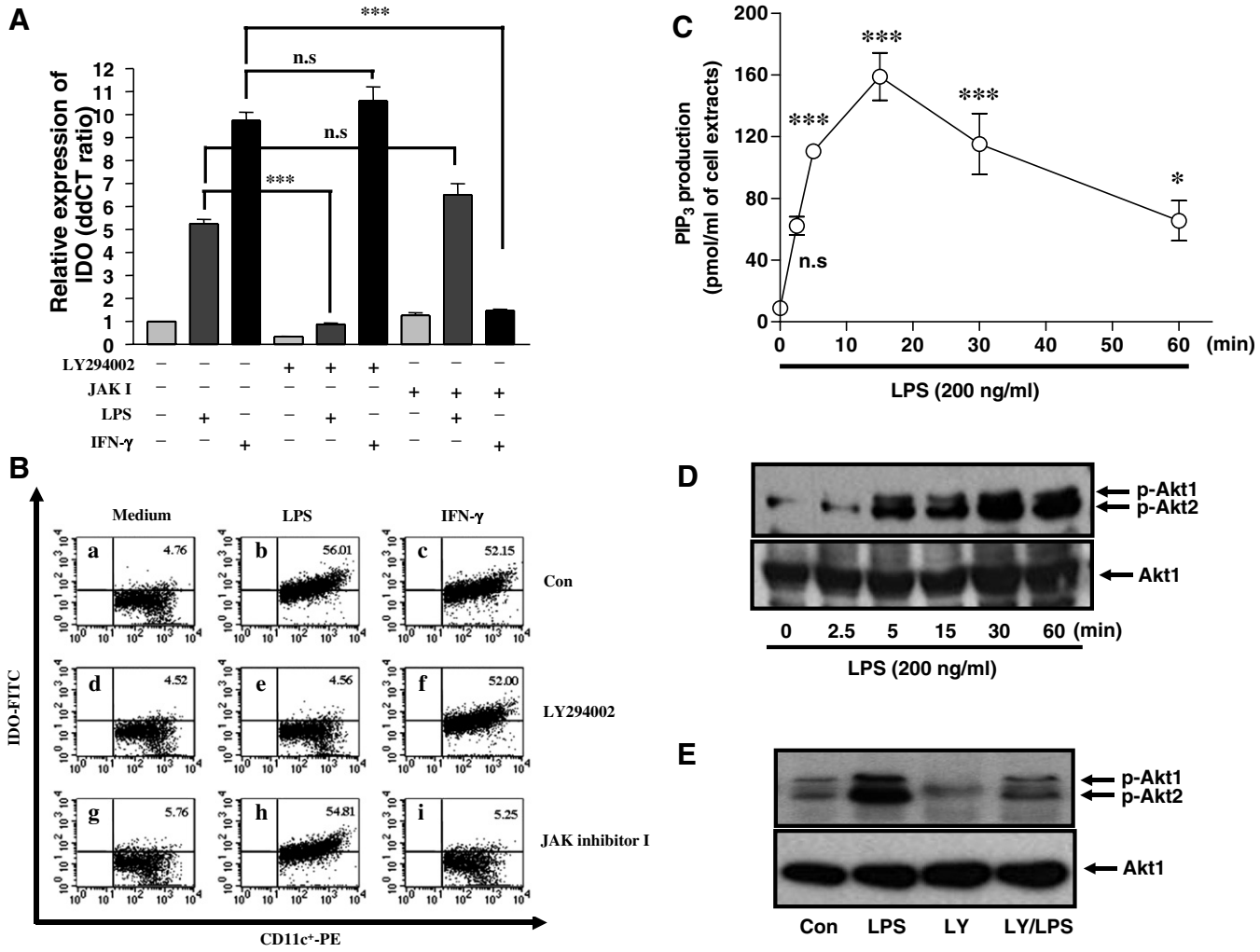

Fig. 2. Effects of PI3K and JAK on LPS- and IFN-γ-induced IDO expression in murine BMDCs. (A) DCs were pre-incubated with LY294002 (10 μM) and JAK inhibitor I (1 μM) for 1 h and further incubated with or without LPS (200 ng/ml) and IFN-γ (100 U/ml) for 24 h, and then cells were harvested and total RNA was extracted. Quantitative mRNA expression was measured as described in Section 2. (B) DCs were pre-incubated with LY294002 (10 μM) and JAK inhibitor I (1 μM) for 1 h and further incubated with or without LPS (200 ng/ml) and IFN-γ (100 U/ml) for 24 h, and then cells were harvested and analyzed by flow cytometry. The cells were gated on $CD11c^+$. (C) DCs were treated with 200 ng/ml LPS for the indicated amounts of time. Enzyme immunoassay of PIP_3 generation by PI3 kinase in DCs extracts as described in Section 2. (D) DCs were treated with 200 ng/ml LPS for the indicated amounts of time. The levels of phosphorylated Akt and total Akt were measured by Western blot analysis with rabbit anti-p-Akt1/2/3 (Thr 308) antibody and mouse anti-Akt1. (E) DCs were pre-incubated with LY294002 (LY, 10 μM) for 1 h and further incubated with or without LPS (200 ng/ml) for 30 min, and then cells were harvested. The levels of phosphorylated Akt and total Akt were measured by Western blot analysis with rabbit anti-p-Akt1/2/3 (Thr 308) antibody and mouse anti-Akt1. Values are means ± S.E.M. obtained from at least three separate experiments. The asterisks in panel **A** indicate significant increases compared to that of LPS- or IFN-γ-treated DCs at $P < 0.001$ (***) and $P > 0.05$ (n.s) and for panel C, asterisks indicate significant increases compared to 0 min at $P < 0.001$ (***), $P < 0.05$ (**) and $P > 0.05$ (n.s).

suggests that JNK is involved in the induction of IDO expression by LPS stimulation (Fig. 3A, *left* and B-e). To further investigate the inhibitory effect of a JNK inhibitor, SP600125, on the expression of IDO induced by LPS, we next assessed the IDO expression in spleenocyte DCs of mice challenged with LPS in vivo. Inbred C57BL/6J mice were injected intraperitoneally with SP600125 (30 mg/kg) for 6 h prior to exposure to LPS, and IDO expression in their spleens was analyzed 18 h later by flow cytometry. We measured the percentage of $IDO^+/CD11c^+$ DCs in the spleen by flow cytometry. As might have been expected, the expression of IDO increased in mice treated with LPS. Similar to the inhibitory effect of SP600125 on the expression of IDO in BMDCs in vitro, SP600125 inhibited IDO expression in $CD11c^+$ DCs of the spleen compared to that of LPS-challenged mice (Fig. 3C).

Next, to test the specificity of MAPK inhibitors such as JNK inhibitor (SP600125), p38 MAPK inhibitor (SB203580) and ERK1/2 inhibitor (U0126), we measured the phosphorylation of JNK, p38 MAPK and ERK1/2 by LPS stimulation in the presence and absence of each inhibitor (Fig. 3D). The phosphorylation of all MAPKs was activated by stimulation with LPS for 30 min. SP600125 inhibited only the phosphorylation of JNK, SB203580 inhibited only the phosphorylation of p38 MAPK and U0126 inhibited the phosphorylation of ERK1/2 indicating that each inhibitor very effectively impaired the activity of each MAPK in our system. These data suggested

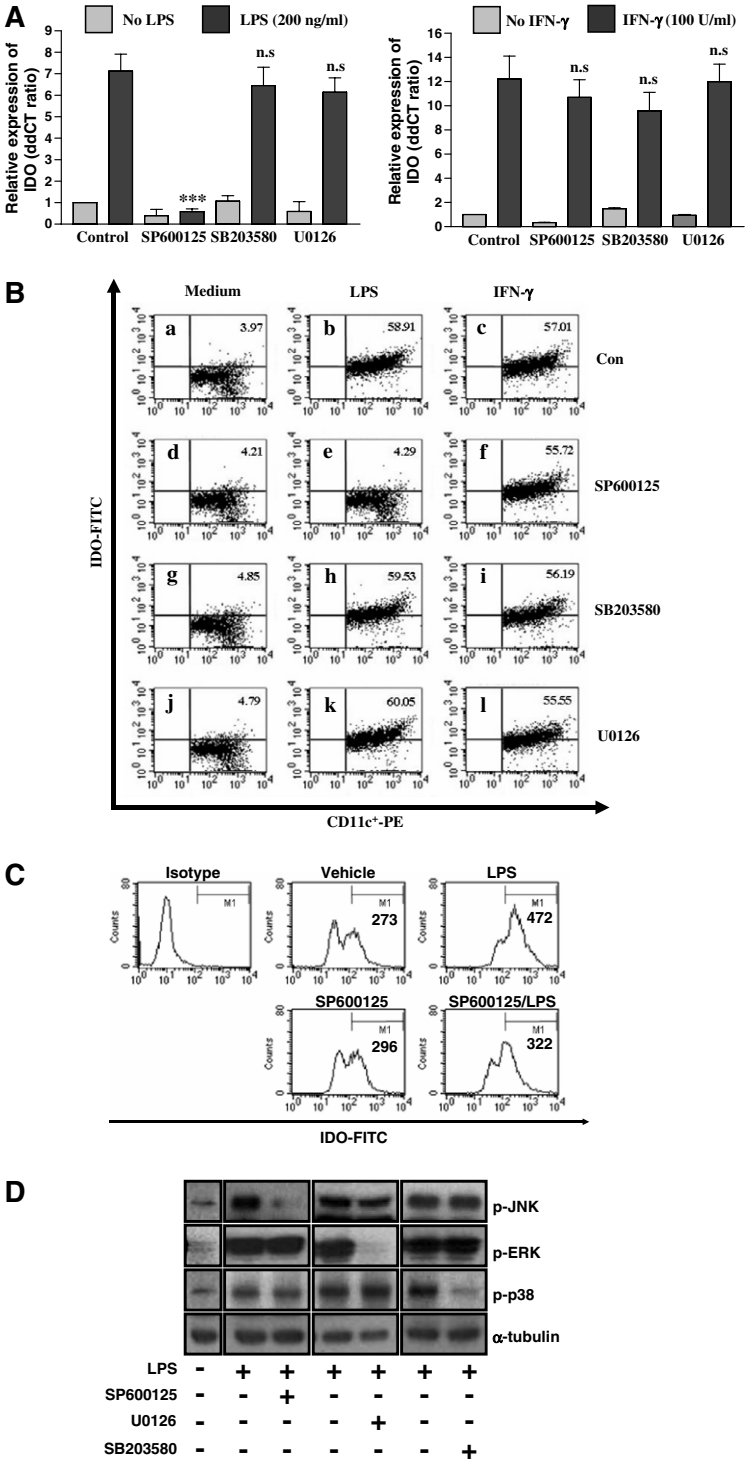

Fig. 3. Effects of MAPK on LPS- and IFN-γ-induced IDO expression in murine BMDCs. (A) DCs were pre-incubated with SP600125 (5 μM), SB203580 (10 μM) and U0126 (4 μM) for 1 h and further incubated with or without LPS (200 ng/ml) and IFN-γ (100 U/ml) for 24 h, and then cells were harvested and total RNA was extracted. Quantitative mRNA expression was measured as described in Section 2. (B) DCs were pre-incubated with SP600125 (5 μM), SB203580 (10 μM) and U0126 (4 μM) for 1 h and further incubated with or without LPS (200 ng/ml) and IFN-γ (100 U/ml) for 24 h, and then cells were harvested and analyzed by flow cytometry. The cells were gated on $CD11c^+$. (C) Before the challenge with LPS, C57BL/6J mice were injected intraperitoneally with SP600125 (30 mg/kg) or DMSO only for 6 h before LPS. And 18 h after the LPS challenge, the spleens from the mice were taken out. Splenic lymphocytes were stained with antibodies for CD11c and IDO. Histogram show expression of intracellular IDO on cells gated for CD11c expression. Numbers indicate the means fluorescence intensity (MFI) values of $CD11c^+$ DCs expressing IDO. (D) DCs were pre-incubated with SP600125 (5 μM), SB203580 (10 μM) and U0126 (5 μM) for 1 h and further incubated with or without LPS (200 ng/ml) for 30 min, and then cells were harvested and total protein was extracted. The levels of phosphorylated JNK, p38 MAPK, ERK1/2 and α-tubulin were measured by Western blot analysis with mouse anti-p-JNK1antibody, rabbit anti-p38 MAPK, mouse anti-ERK and rabbit anti-α-tubulin antibody. Values are means ± S.E.M. obtained from at least three separate experiments. The asterisks in panel A indicate significant increases compared to that of LPS- or IFN-γ-treated DCs at $P < 0.001$ (***) and $P > 0.05$ (n.s).

that LPS-induced IDO expression was mediated by JNK in DCs.

3.4. The relationship between PI3K and JNK/c-Jun in DCs

To determine the relationship between PI3K and JNK/c-Jun in this signaling pathway, we measured the level of JNK and c-Jun phosphorylation by flow cytometry. Fig. 4A shows the time course of activation of the JNK and c-Jun in BMDCs treated with LPS for indicated amounts of time. The phosphorylation of JNK and c-Jun by LPS was stimulated within 5 min (Fig. 4A-b,g) and slightly increased in stimulation after approximately 30 min. We also measured the phosphorylation of JNK induced by LPS stimulation, as analyzed by Western blot to avoid non-specific binding (Fig. 4B). LPS stimulated the phosphorylation of JNK in a time-dependent manner similar to that shown in Fig. 4A. Next, to investigate whether PI3K is involved in LPS-induced activation of JNK and c-Jun, we measured the level of JNK and c-Jun phosphorylation after LPS stimulation for 30 min in the presence and absence of LY294002. As shown in Fig. 3B, LPS-induced JNK and c-Jun phosphorylation (Fig. 4C-b,f) were significantly down-regulated by LY294002 (Fig. 4C-d,h), indicating that PI3K is an upstream molecule of JNK.

Two unrelated products, wortmannin and LY294002, are extensively used as pharmacological agents for characterizing the role of PI3K in cellular signaling [26]. Wortmannin is a potent, cell-permeable, potent, selective inhibitor of PI3K (IC_{50} = 50 nmol/L) and pleckstrin phosphorylation [27]. The quercetin derivative LY294002 (2-(4-morphlinyl)-8-phenyl-4H-1-benzopyran-4-one), is a specific and cell-permeable inhibitor of PI3K but is less potent (IC_{50} = 50 μmol/L) than wortmannin [28]. Therefore, we measured the phosphorylation of JNK by LPS stimulation in the presence of PI3K inhibitors, wortmannin (500 nM) and LY294002 (10 μM), as analyzed by Western blot to avoid non-specific binding (Fig. 4D). These data suggest that LPS-induced IDO gene expression may be modulated by PI3K-JNK/c-Jun signaling pathways in BMDCs.

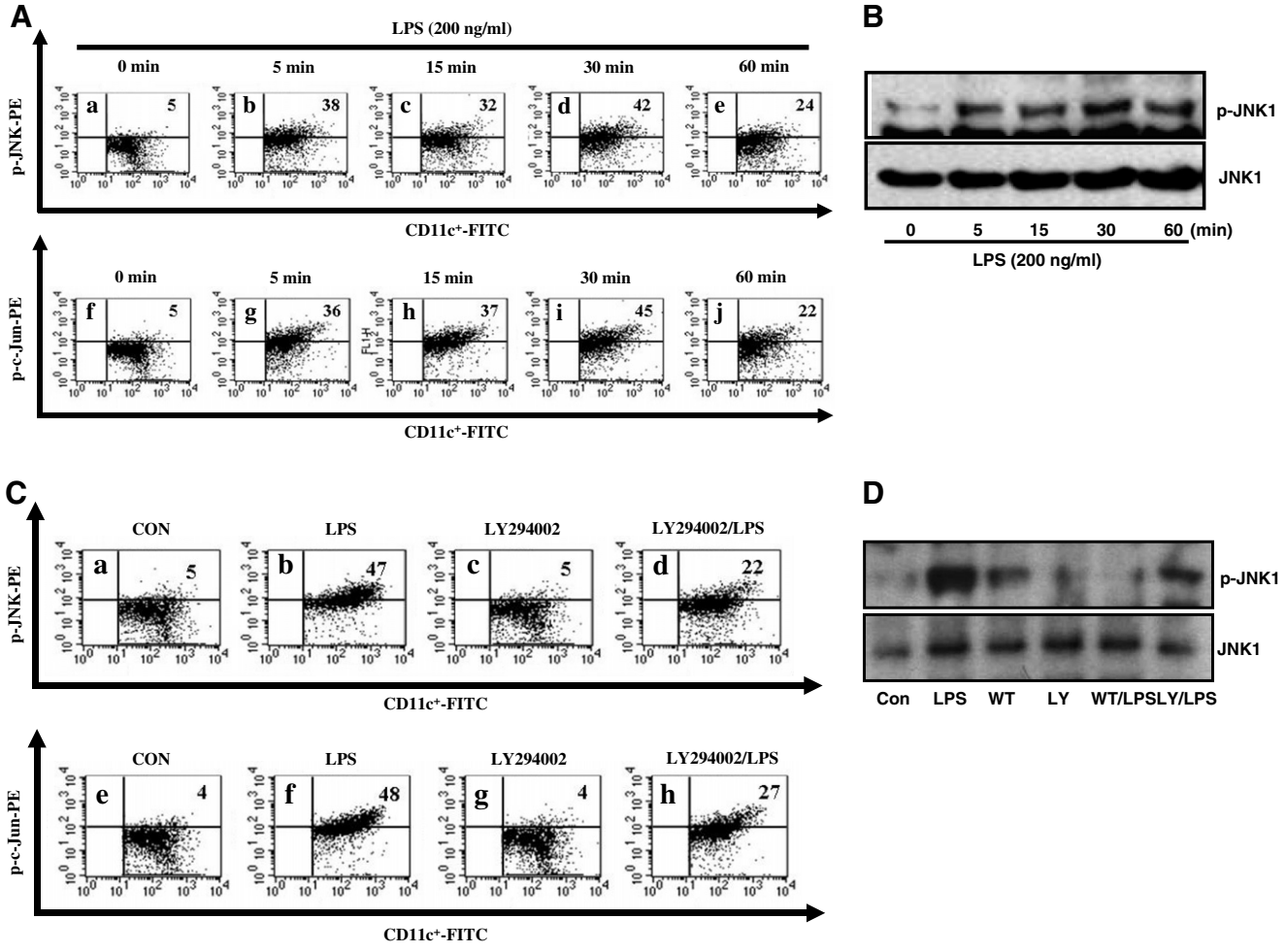

Fig. 4. Relationship between PI3K and JNK on LPS-induced signaling pathway. DCs were treated with LPS (200 ng/ml) for indicated time points. (A) The cells were harvested and analyzed by flow cytometry. The cells were gated on $CD11c^+$. (B) The levels of phosphorylated JNK and total JNK were measured by Western blot analysis with mouse anti-p-JNK1antibody and rabbit anti-JNK1 antibody. (C) DCs were pre-incubated with LY294002 (10 μM) for 1 h and further incubated with or without LPS (200 ng/ml) for 30 min, and then cells were harvested and analyzed by flow cytometry. The cells were gated on $CD11c^+$. (D) DCs were pre-incubated with wortmannin (WT, 500 nM) and LY294002 (LY, 10 μM) for 1 h and further incubated with or without LPS (200 ng/ml) for 30 min, and then cells were harvested. The levels of phosphorylated JNK and total JNK were measured by Western blot analysis with mouse anti-p-JNK1antibody and rabbit anti-JNK1 antibody. Values are means ± S.E.M. obtained from at least three separate experiments.

4. Discussion

In this study, we have identified a novel signaling pathway in LPS-stimulated murine BMDCs inducing the gene expression of IDO. Our data showed that the activation of PI3K and JNK/c-Jun is necessary for LPS-induced IDO gene expression.

Regulation of IDO expression can be mediated by several cytokines and immunomodulating agents including TNF-α, IL-1 and LPS. Each of these agents can enhance the amount of IDO activity induced in IFN-γ-treated macrophages in the THP-1 cell line [29,30]. Babcock and Carlin [31] reported that IFN-γ with IL-1 or TNF-α enhances the activity of IDO through the transcriptional activity of the IDO promoter region. Previous reports demonstrated that both IL-2 and IL-12 increased the IDO via an IFN-γ dependent mechanism [32,33]. On the other hand, the induction of IDO was prevented by IL-4 and TNF-α in human cell lines [34,35]. Very recent studies have suggested that the induction of IDO by LPS is regulated by an IFN-γ-independent mechanism and may be related to the activity of the p38 MAPK pathway and NF-κB [36].

Although the differences in the signaling pathways between LPS and IFN-γ on IDO gene expression remain largely unknown, our findings indicated that LPS-induced IDO expression is mediated by PI3K and JNK whereas IFN-γ-induced IDO expression is regulated by JAK. These results suggest that LPS and IFN-γ belong to different signaling pathway responses of IDO induction in BMDCs. Furthermore, we intended to demonstrate the possible basis of LPS or IFN-γ-induced IDO gene expression through the alteration of the cellular expression levels of JNK, p38 MAPK, and ERK1/2. Our study demonstrates that only the JNK inhibitor, SP600125, significantly inhibits IDO gene expression in LPS-stimulated BMDCs (Fig. 3A and B). The biological roles of JNKs have been extremely difficult to demonstrate due to a lack of specific pharmacologic inhibitors.

To further confirm the results of our in vitro study (Fig. 3A and B), we employed one strategy to determine whether LPS-induced IDO expression was dependent on JNK activation. We used the available specific anthrapyrazolone JNK inhibitor SP600125, a reversible ATP-competitive inhibitor with greater than 20-fold selectivity compared to other kinases in inhibiting the action of phospho-JNK but not the expression of phospho-JNK [21]. Using SP600125, we found that administration of SP600125 in mice in vivo decreased the expression of IDO by LPS in spleenocyte dendritic cells (Fig. 3C). However, none of the MAPKs inhibitors affected IFN-γ-induced IDO expression (Fig. 3B).

Since PI3K has been shown to be crucial for in JNK phosphorylation in other cell types after Fc receptor cross-linking [37] or upon exposure to platelet-derived growth factor [38], angiotensin II [39], and LPS [23], we found that the PI3K inhibitors, wortmannin and LY294002, significantly inhibited the phosphorylation of JNK and c-Jun by LPS stimulation (Fig. 4C and D). Taken together, these results suggest that only JNK among the MAPKs is strongly involved in LPS-induced IDO gene expression, and indicated that JNK/c-Jun pathways induced by LPS are modulated by PI3K. Although LPS-induced IDO expression was completely inhibited by both LY294002 and SP600125, LY294002 partially inhibited LPS-induced JNK activation indicating that LPS-induced IDO gene expression via the activation of JNK could be mediated by a PI3K-dependent or -independent pathway. It has been demonstrated that RNA-dependent protein kinase (PKR) mediates the phosphorylation of JNK by specific proinflammatory stress stimuli such as LPS, IL-1β, and TNF-α [40]. The innate immune response is initiated by the recognition of invariant molecular components of infectious agents such as LPS and double-strand RNA (dsRNA), a common intermediate of viral replication [41]. While the receptors for LPS are extracellular (CD14) and transmembrane proteins (TLR4) [42], dsRNA only binds to intracellular targets, including PKR. Upon binding to dsRNA, PKR is activated by autophosphorylation, and subsequently phosphorylates the α-subunit of eukaryotic initiation factor 2 (eIF-2α), thereby inhibiting translation [43]. Further studies are required to better understand the detailed molecular mechanism of IDO gene expression including the PKR-dependent pathway induced by LPS in BMDCs.

In summary, we have determined the mechanism by which LPS induces the expression of IDO in murine BMDCs. We show that the inhibition of the PI3K and JNK signaling pathways is implicated in LPS-induced IDO gene expression, and that LPS-induced IDO expression is mediated by an IFN-γ-independent mechanism in murine BMDCs.

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