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The Signal Transducer and Activator of Transcription 1α and Interferon Regulatory Factor 1 Are Not Essential for the Induction of Indoleamine 2,3-Dioxygenase by Lipopolysaccharide: Involvement of p38 Mitogen-Activated Protein Kinase and Nuclear Factor-κB Pathways, and Synergistic Effect of Several Proinflammatory Cytokines

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Indoleamine 2,3-dioxygenase (IDO) is induced by interferon (IFN)-γ–mediated effects of the signal transducer and activator of transcription 1α (STAT1α) and interferon regulatory factor (IRF)-1. The induction of IDO can also be mediated through an IFN-γ–independent mechanism, although the mechanism of induction has not been identified. In this study, we explored whether lipopolysaccharide (LPS) or several proinflammatory cytokines can induce IDO via an IFN-γ–independent mechanism, and whether IDO induction by LPS requires the STAT1α and IRF-1 signaling pathways. IDO was induced by LPS or IFN-γ in peripheral blood mononuclear cells and THP-1 cells, and a synergistic IDO induction occurred when THP-1 cells were cultured in the presence of a combination of tumor necrosis factor-α, interleukin-6 or interleukin-1β. An electrophoretic mobility shift assay using STAT1α and IRF-1 consensus oligonucleotide probes showed no STAT1α or IRF-1 binding activities in LPS-stimulated THP-1 cells. Further, the LPS-induced IDO activity was inhibited by both p38 mitogen–activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) inhibitors. These findings suggest that the induction of IDO by LPS in THP-1 cells is not regulated by IFN-γ *via* recruitment of STAT1α or IRF-1 to the intracellular signaling pathway, and may be related to the activity of the p38 MAPK pathway and NF-κB.

Key words: enzyme induction, indoleamine 2,3-dioxygenase, interferon regulatory factor-1, lipopolysaccharide, signal transducer and activator of transcription 1α.

Abbreviations: Ab, antibody; EMSA, electrophoretic mobility shift assay; IDO, Indoleamine 2,3-dioxygenase; IFN, interferon; ISRE, interferon-stimulated response elements; IL, interleukin; IRF, interferon regulatory factor; GAS, IFN-γ–activated site; L-KYN, L-Kynurenine; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PBMC, peripheral blood mononuclear cells; STAT1α, signal transducer and activator of transcription 1α; TNF, tumor necrosis factor; Trp, tryptophan.

Indoleamine 2,3-dioxygenase (IDO) is the rate-limiting and first enzyme in the L-tryptophan-kynurenine pathway that converts the essential amino acid L-tryptophan (L-Trp) to *N*-formylkynurenine in mammalian extrahepatic tissues. IDO activities in several tissues are highly induced under various pathological conditions including tumor regression, allograft rejection, cerebral ischemia and viral or bacterial infection (*1*–*3*). While several studies have circumstantially linked IDO induction to certain of the antiproliferative and anti-microbial effects of immune stimuli (*4*–*6*), different and potentially detrimental consequences of IDO induction have also been proposed (*3*, *7*). IDO induction can deplete L-Trp, which exists as the least abundant of all essential amino acids, in target cells, and this is partially responsible for the anti-microbial, anti-viral, and anti-proliferative activities of interferon (IFN)-γ (*6*, *8*). L-Trp depletion is also involved in the inhibition of T cell proliferation by IFN-γ–treated human monocyte–derived macrophages and dendritic cells (*9*). Further, there have been several reports that the activation of L-arginine metabolism through inducible nitric oxide synthase induction leads to the formation of nitric oxide and peroxynitrite, which in turn, down-regulate L-Trp metabolism by inhibiting IDO (*10*, *11*). On the other hand, several metabolites of the L-tryptophan-kynurenine pathway act to induce excitotoxic, neuronal death and may cause apoptosis (*12*).

It is widely accepted that interferons, particularly IFN-γ, are essential factors for IDO induction, because two interferon-stimulated response elements (ISREs)

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and IFN-γ–activated site (GAS) element sequences are found in the 5′-flanking region of the IDO gene, and IFN-γ actually induces IDO in many cells (*13–15*). However, previous studies have suggested that IDO induction may also be mediated by an IFN-γ–independent mechanism under certain circumstances (*16–18*). In fact, infiltrating macrophages and microglia are considered to be the origin of several inflammatory cytokines and IDO (*19*, *20*). Further, a recent study demonstrated that interferon regulatory factor (IRF)-8 regulates IDO expression in monocyte-derived dendritic cells (*21*). In this study, we explored whether lipopolysaccharide (LPS) can induce IDO *via* an IFN-γ–independent mechanism, and whether IDO induction by LPS requires the signal transducer and activator of transcription 1α (STAT1α) and IRF-1 signaling pathways in THP-1 cells.

MATERIALS AND METHODS

Materials—Human recombinant IFN-γ (specific activity = 2.0×10^7 U/mg), tumor necrosis factor (TNF)-α (specific activity = 1.0×10^8 U/mg), interleukin (IL)-1β (specific activity = 5.0×10^7 U/mg), IL-6 (specific activity = 1.0×10^8 U/mg), anti-TNF-α antibody (Ab) (50 ng of antibody neutralizes 1 U TNF-α), anti–IL-1β Ab (250 ng of antibody neutralizes 1 U IL-1β), anti–IL-6 Ab (20 ng of antibody neutralizes 1 U IL-6), and anti–IFN-γ Ab (200 ng of antibody neutralizes 1 U IFN-γ) were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany). LPS (from Salmonella abortus equi) and L-Kynurenine (L-KYN) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). SB202190, SB202474, SN50 and SN50M were from Calbiochem (San Diego, CA, USA). All other chemicals of analytical grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Culture Conditions—THP-1 (human acute monocyte leukemia cell line) and MRC9 (human lung cell line) were obtained from Health Science Research Resources Bank (Osaka Japan); SK-HEP-1 (human liver adenocarcinoma), U937 (human monocyte, histiocytic lymphoma) and SK-N-SH (human brain neuroblastoma) were obtained from American Type Culture Collection (Manassas, VA, USA); and peripheral blood mononuclear cells (PBMC) from human volunteers were obtained from ABI (Advanced Biotechnologies Inc., Columbia, MD, USA). THP-1 and U937 cells were conventionally cultivated in RPMI 1640 (Nikken Bio Medical Laboratory, Kyoto, Japan) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Boehringer Mannheim Biochemica) and 100 U/ml of penicillin-streptomycin (Invitrogen Corporation, Carlsbad, CA, USA). PBMC, MRC-9, SK-HEP-1 and SK-N-SH cells were cultivated in Dulbecco's minimum essential medium supplemented with 10% FBS, and harvested with trypsin-EDTA as described previously (*22*). Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were treated with LPS or various cytokines as described in the figure legends. After treatment, an aliquot of culture media was used for the determination of L-KYN concentration, and cell pellets were collected by centrifugation after washing with phosphate-buffered saline.

Measurement of TNF-α, IL-6, IL-1β, IFN-γ and L-KYN—The concentrations of TNF-α IL-6, IL-1β and IFN-γ released by THP-1 cells into the culture supernatant were measured by ELISAs as described previously (*23*). L-KYN concentrations were measured by HPLC as described previously (*16*).

Preparation of Nuclear Extract—THP-1 cells were collected and nuclear extracts were prepared as described previously (*24*), then frozen and stored at –80°C until use. All solutions used for the preparation of nuclear extracts contained protease inhibitors. Protein concentrations were measured by the Bradford method (BioRad Laboratories, Richmond, CA, USA) with bovine serum albumin as the standard.

Electrophoretic Mobility Shift Assay (EMSA)—STAT1α and IRF-1 consensus or mutated oligonucleotide probes were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The probes were end-labeled with γ^{32}P-ATP using T4 polynucleotide kinase (Invitrogen Corporation, Carlsbad, CA, USA). A mixture of 10 μg of nuclear protein and 0.2 μg of ^{32}P end-labeled double-stranded oligonucleotide probe was incubated for 30 min at room temperature, then electrophoresed in a 3% polyacrylamide tris-glycine-EDTA gel. Specific antibodies were purchased from Santa Cruz Biotechnology and were used in gel supershift analysis as follows: 0.2 μg of each anti-STAT1α p91 (C-24) or anti–IRF-1 (M-20) antibody was added and incubated for 30 min at room temperature before electrophoresis. The gels were dried and Kodak X-AR film (Rochester, NY, USA) was exposed to the gel for a period of 2 h to 2 days at –80°C.

Treatment for NF-κB and p38 MAPK Inhibitor—SN50 (40 μM), which inhibits the translocation of the NF-κB active complex into the nucleus, with its inactive control peptide SN50M (40 μM), along with SB202190 (12 μM), a potent and specific inhibitor of p38 MAPK, and SB202474 (12 μM), an inactive analog of SB202190, were added before treatment with LPS or IFN-γ. After 30 min, 1 μg/ml LPS or 5 ng/ml IFN-γ was added and the mixtures were incubated

Table 1. **IDO induction by LPS or IFN-γ in several human cell lines.** Each cell line was stimulated with 1 μg/ml of LPS or 5 ng/ml of IFN-γ for 24 h, and L-KYN concentrations in the culture supernatant were measured. All baseline L-KYN concentrations in the culture medium were subtracted. Values are means ± SD of three separate experiments. *$p < 0.01$ compared with each control.

Cell line	L-KYN (μM)		
	Control	LPS	IFN-γ
PBMC (peripheral blood mononuclear cell)	0.20 ± 0.06	27.04 ± 2.40*	27.63 ± 1.24*
THP-1 (monocyte; acute monocytic leukemia)	0.15 ± 0.02	23.55 ± 0.97*	14.39 ± 0.36*
U937 (monocyte; histiocytic lymphoma)	2.83 ± 0.08	30.50 ± 0.86*	11.63 ± 0.48*
SK-HEP-1 (liver; ascites; adenocarcinoma)	0.40 ± 0.07	0.65 ± 0.12	66.40 ± 1.38*
MRC9 (lung; fibroblast)	0.08 ± 0.07	0.35 ± 0.17	61.60 ± 1.38*
SK-N-SH (brain; neuroblastoma)	0.04 ± 0.01	0.10 ± 0.05	40.96 ± 0.69*

for 24 h. The culture supernatants were collected and L-KYN concentrations were measured.

Statistical Analyses—Results are expressed as means ± SD. Intergroup comparisons were made using one-way analysis of variance (ANOVA), followed by Scheffe's F post-hoc test. A p value less than 0.05 was considered statistically significant.

RESULTS

IDO Induction by LPS Is Unique in Monocyte/Macrophage Cells—Table 1 shows IDO induction by LPS or IFN-γ in several human cell lines. LPS significantly induced IDO activity in PBMC, THP-1 and U937 cell lines as compared with the other cell lines. In contrast, IFN-γ which is a known dominant inducer of IDO in various cell lines, induced IDO activity in all cell lines tested. These results indicate that IDO is much more responsive to LPS in monocyte/macrophage-like cells than in non-monocyte/macrophage cells.

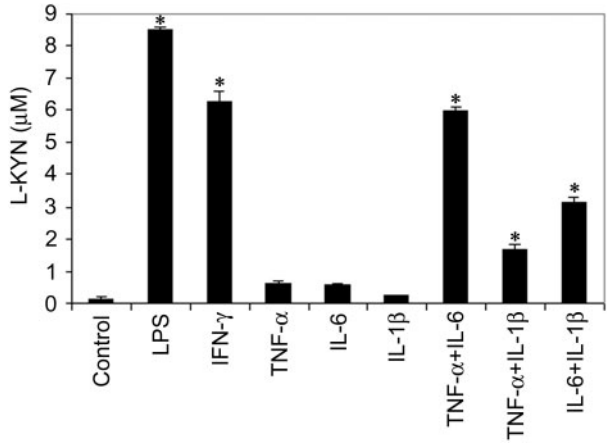

Fig. 1. **Increased L-KYN levels in THP-1 cells stimulated by LPS or various proinflammatory cytokines.** THP-1 cells were treated with LPS (1 μg/ml), IFN-γ (5 ng/ml) TNF-α (1 ng/ml), IL-6 (1 ng/ml) or IL-1β (2 ng/ml) alone or in combination with TNF-α and IL-6 (1 ng/ml respectively), TNF-α and IL-1β (1 ng/ml and 2 ng/ml, respectively) or IL-6 and IL-1β (1 ng/ml and 2 ng/ml, respectively). The cells were cultured for 24 h with cytokines, and L-KYN concentrations in the culture supernatants were measured. Each bar represents the mean ± SD of the results from five separate experiments. *$p < 0.005$ compared with control.

LPS-Induced IDO and the Synergistic Induction of IDO by TNF-α, IL-6 or IL-1β in THP-1 Cells—IDO was induced by LPS (1 μg/ml) or IFN-γ (5 ng/ml) in THP-1 cells, and a significant synergistic IDO induction occurred when THP-1 cells were cultured in the presence of a combination of TNF-α (1 ng/ml), IL-6 (1 ng/ml) or IL-1β (2 ng/ml), although TNF-α, IL-6 or IL-1β alone did not induce IDO in THP-1 cells (Fig. 1). However, the non-macrophage/monocyte cell lines MRC9 (human lung fibroblast), H4 (human brain neuroglioma), and SK-N-SH (human brain neuroblastoma) were negative for IDO induction by any combination of these cytokines or LPS (data not shown). These data indicate that THP-1 cells are able to induce IDO by several proinflammatory cytokines with the exception of IFN-γ, and that IDO is up-regulated by combination with TNF-α, IL-6 or IL-1β in a synergistic manner in THP-1 cells.

LPS Induction of IDO Mediated by STAT1α- or IRF-1–Independent Mechanisms in THP-1 Cells—STAT1α and IRF-1 binding activities that induce IDO transcriptional activity by IFN-γ in THP-1 cells were tested by EMSA (Fig. 2, A and B). The super-shifted band appeared only in IFN-γ–stimulated THP-1 cells and not in LPS-stimulated THP-1 cells, and these results were also observed in time-point experiments from 30 min to 24 h following LPS treatment (data not shown). The control and LPS-stimulated extracts gave protein-DNA complexes with the same mobility as the STAT1α or IRF-1 complex. However, the assay using oligonucleotide probes with each mutated binding site showed that non-specific

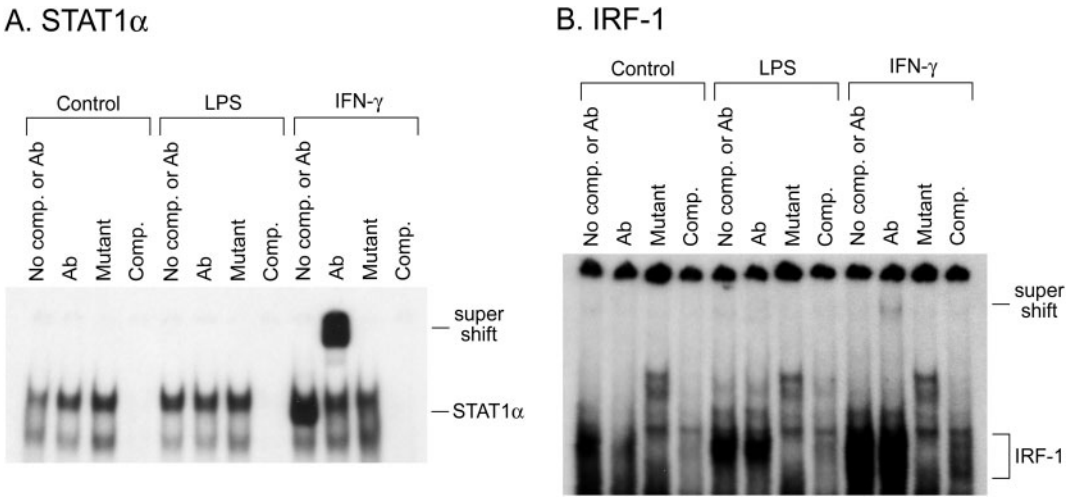

Fig. 2. **Electrophoretic gel mobility shift assay (EMSA) for the activation of STAT1α (A) and IRF-1 (B) in THP-1 cells.** EMSA was performed as described in "MATERIALS AND METHODS." Nuclear extracts were prepared from THP-1 cells stimulated for 30 min (A) or 4 h (B) with 1 μg/ml of LPS or 5 ng/ml of IFN-γ, and used in bandshift analyses with oligonucleotide probes for either the STAT1α (A) or IRF-1 (B) consensus sequence or mutated oligonucleotide probes (mutant). Following binding to the probes, anti-STAT1α (A) or anti–IRF-1 (B) antibodies were added to the binding reactions (Ab). A 100-fold molar excess of unlabeled STAT1α (A) or IRF-1 (B) probe was used as a competitor (comp.). Shown are the results of one of three analyses with similar results.

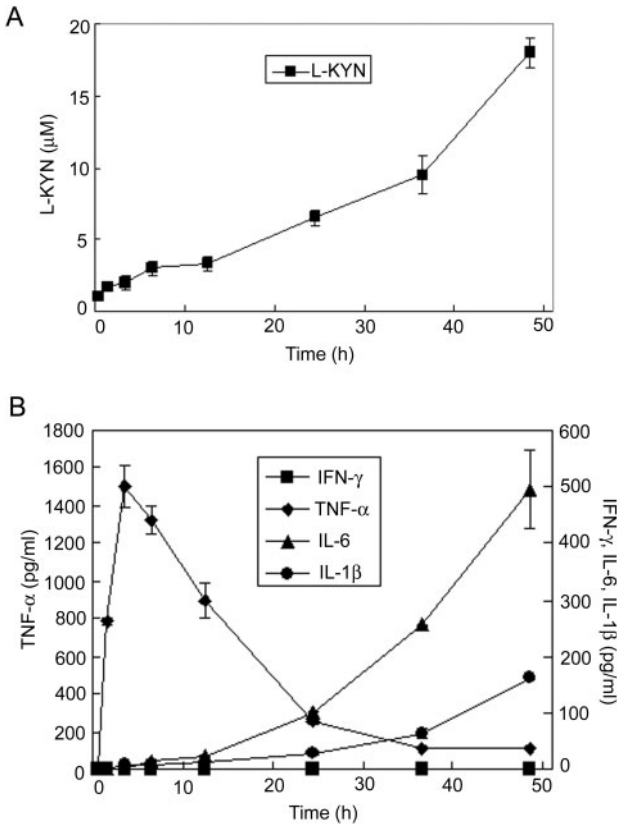

Fig. 3. **Time course of the release of L-KYN, IFN-γ, TNF-α, IL-6, and IL-1β into culture supernatant of THP-1 cells following LPS stimulation.** THP-1 cells were incubated in the presence of 1 μg/ml of LPS for the indicated time periods. At the times indicated, the culture supernatant was collected and L-KYN concentration was measured by HPLC (A) and IFN-γ, TNF-α, IL-6 and IL-1β protein concentrations were measured by ELISA (B). Each bar represents the mean ± SD of the results of three separate analyses.

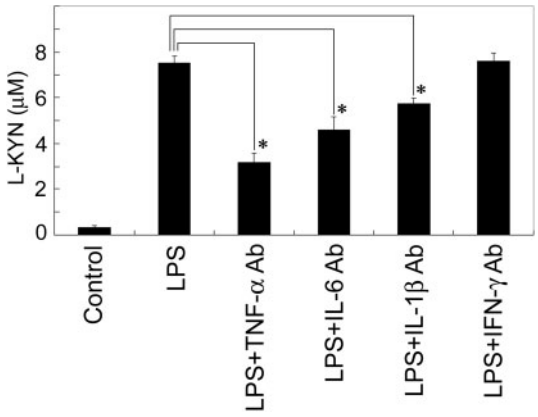

Fig. 4. **Effect of various proinflammatory cytokine antibodies on L-KYN production in LPS-stimulated THP-1 cells.** THP-1 cells were cultured for 48 h in the absence or presence of LPS treated with anti hTNF-α Ab (10 μg/ml) or anti hIL-6 Ab (10 μg/ml) or anti hIL-1β (25 μg/ml) or anti hIFN-γ Ab (20 μg/ml). The antibody was added with the LPS, and samples were collected 48 h later. *$p < 0.005$ compared with LPS-treated cells.

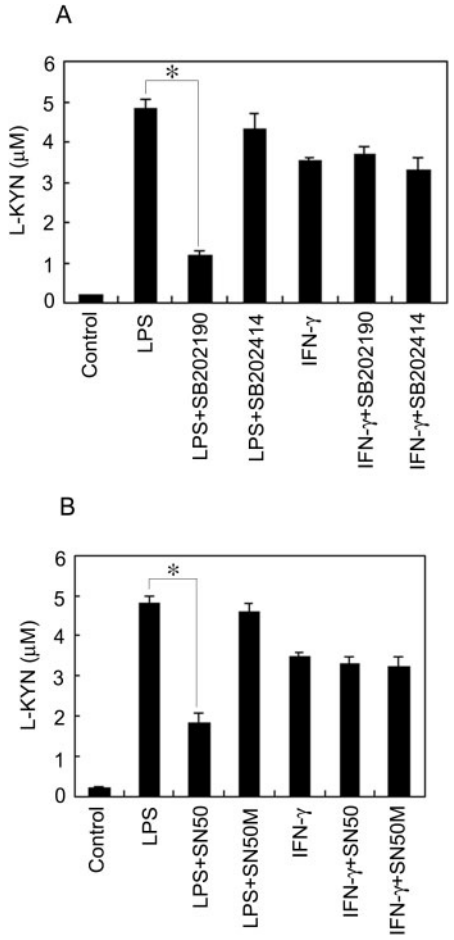

Fig. 5. **Effect of SN50 and SB202190 on the induction of IDO by LPS.** THP-1 cells were pre-treated (30 min) with 12 μM of SB202190 or SB202414 (A), or with 40 μM of SN50 or SN50M (B), and exposed to 1 μg/ml of LPS or 5 ng/ml of IFN-γ for 24 h, after which L-KYN concentrations in culture supernatants were measured. Each bar represents the mean ± SD of the results of three separate analyses. *$p < 0.005$ compared with control.

binding existed with the same mobility as the STAT1α or IRF-1 complex. In addition, Abs against IRF-1 did not deplete all the complexes, suggesting the presence of complexes with other IRF family members. Thus, these EMSA analyses show that binding activities of STAT1α and IRF-1 were absent in THP-1 cells stimulated by LPS. These results indicate that the induction of IDO by LPS is mediated by STAT1α- and IRF-1–independent mechanisms in THP-1 cells.

Time Course of IDO and Cytokine Induction after Exposure of THP-1 Cells to LPS—The time course of IDO induction and release of several pro-inflammatory cytokines into the culture supernatant by THP-1 cells after treatment with 1 μg/ml of LPS is shown in Fig. 3. L-KYN concentration increased in a time-dependent manner (Fig. 3A). Figure 3B shows the release of IFN-γ, TNF-α, IL-6 and IL-1β into the culture supernatant by THP-1 cells after treatment with LPS. The TNF-α concentration increased significantly to a maximum at 3 h following LPS treatment, but thereafter rapidly declined. IL-6 and IL-1β increased from 24 to 36 h following LPS treatment,

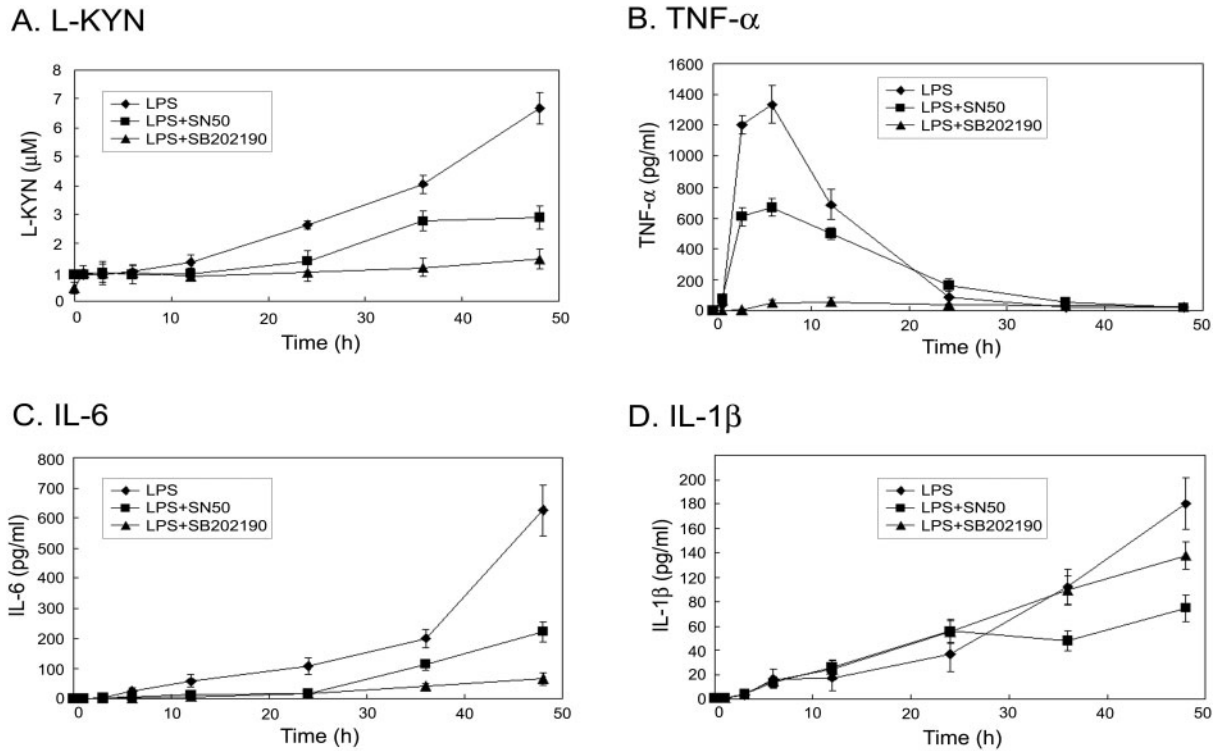

Fig. 6. **Effect of SB202190 and SN50 on L-KYN, TNF-α, IL-1β and IL-6 production by LPS-stimulated THP-1.** THP-1 cells were incubated in the presence of 1 μg/ml of LPS in the absence or presence of SB202190 (12 μM) or SN50 (40 μM) treatment for the indicated time periods. At the times indicated, the culture supernatant was collected and L-KYN concentration was measured by HPLC (A), and TNF-α (B), IL-6 (C) and IL-1β (D) protein concentrations were measured by ELISA.

and the increases continued until 48 h. No IFN-γ protein was detectable at any time (Fig. 3B), although THP-1 cells normally responded to LPS according to the expression of other pro-inflammatory cytokines. These results suggest that LPS-induced IDO is independent of induction by the IFN-γ signaling pathway.

It is known that macrophages activated by LPS release several proinflammatory cytokines, and that the cytokines activate the macrophage itself by an autocrine mechanism. The possible involvement of these cytokines in LPS-mediated IDO induction was examined in THP-1 cells using neutralizing Abs against the cytokines. The results revealed the involvement of TNF-α, IL-6, and IL-1β in the IDO induction because the addition of each Ab significantly suppressed the production of L-KYN (Fig. 4).

IDO Induction by LPS Is Related to the Activities of p38 MAPK and NF-κB—To investigate which signaling pathway is related to LPS-induced IDO, we also examined the possible roles of p38 MAPK and NF-κB in the induction of IDO by LPS in THP-1 cells. As shown in Fig. 5, SB202190 (a p38 MAPK inhibitor) and SN50 (an NF-κB inhibitor) significantly inhibited LPS-induced IDO. In contrast, SB202190 and SN50 did not inhibit IFN-γ–induced IDO. These results suggest that LPS-induced IDO is related to the activity of the p38 MAPK pathway and NF-κB. Since there is a possibility that p38MAPK and NF-κB inhibitors could affect TNF-α, IL-6 and IL-1β production, these cytokines were measured in the culture supernatant after treatment with 1 μg/ml of LPS and SB202190 or SN50 (Fig. 6). The results revealed that p38 MAPK and NF-κB inhibitors partially suppressed LPS-induced TNF-α, IL-6 and IL-1β production in THP-1 cells.

DISCUSSION

This study demonstrates that IDO induction by LPS is not mediated by STAT1α or IRF-1 binding activities that induce IDO transcriptional activity by IFN-γ in many cells. To date, it is known that IDO is up-regulated by IFN-γ–dependent and/or –independent mechanisms, and down-regulated by IL-4 and transforming growth factor-β (*16*, *25*, *26*). Recent studies have suggested that IDO expressed by macrophage- and/or monocyte-derived dendritic cells has regulatory effects on T cells that result from tryptophan depletion in specific local tissue microenvironments (*9*, *27*). However, it has not yet been established whether the physiological role of IDO regulation by cytokines and immunomodulating agents is beneficial or detrimental.

Table 1 shows that IDO in human PBMC, U937 and THP-1 cells is induced by LPS, although non-monocyte/macrophage cell lines, SK-HEP-1, H4, MRC9 and SK-N-SH cell lines, show no induction of IDO by LPS. These results indicate that human monocyte/macrophage cells and certain other cells may have particular mechanisms for the induction of IDO by LPS or several inflammatory cytokines other than IFN-γ. Because IDO expressed by monocyte-derived macrophges or dendritic cells has inhibitory effects on T cell proliferation, IDO induction by LPS or other inflammatory cytokines as well as IFN-γ may play an important role in T cell function. Indeed, a recent

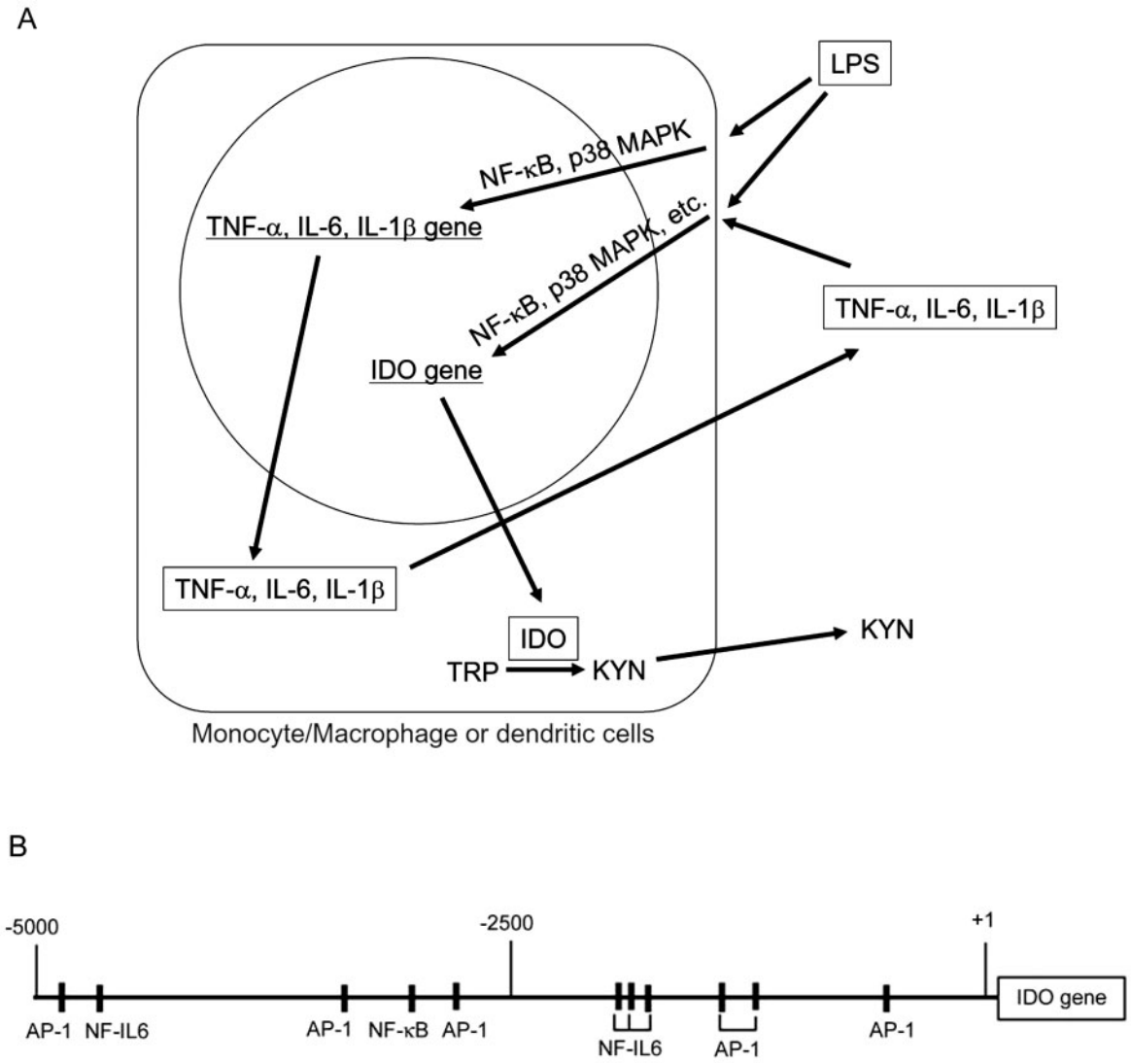

Fig. 7. **A schematic overview of IDO induction by LPS (A) and the location of potential regulatory elements such as AP-1, NF-κB and NF-IL-6 on the human IDO gene promoter region (B).** A homology search of the human IDO promoter region was performed using DNASIS software (Hitachi Software Engineering, Tokyo, Japan). The sequence of the IDO promoter region was deposited in the Genbank database (accession number; NT_008251).

study suggested that IDO-expressed mononuclear cells in patients with inflammatory bowel disease are inducible by TNF-α (*28*). LPS stimulation of human monocytes and macrophages activates several intracellular signaling pathways including the IkappaB kinase–NF-κB pathway and MAPK pathways. These pathways in turn activate a variety of transcription factors that include NF-κB and activator protein-1 (AP-1). LPS-responsive *cis*-acting DNA promoter elements have been characterized in the 5′-flanking region of many genes encoding inflammatory mediators. The IDO gene has 2 ISREs and 3 GAS element that are required for the induction of IDO activity by IFN-γ (*13–15*). Figure 2 shows that STAT1α and IRF-1 binding activities are not induced in LPS-treated THP-1 cells. In addition, IDO induction by LPS in THP-1 cells was significantly attenuated by the addition of NF-κB inhibitor and p38 MAPK inhibitor, but IDO induction by IFN-γ was not affected (Fig. 5). These results suggest that part of the induction of IDO by LPS may be mediated by a signal from the NF-κB or p38MAPK pathway, and that this signaling may not be required for IDO induction by IFN-γ. Further, this study shows that TNF-α, IL-6 and IL-1β are produced by LPS-stimulated THP-1, and that the induction of IDO by LPS is mediated through the production of these cytokines. It is likely that these cytokines regulate IDO transcriptional activity after LPS-treatment. A homology search of the 5′-flanking region of the IDO gene using DNASIS software (Hitachi Software Engineering, Tokyo, Japan) shows consensus sequences for transcriptional factors, such as AP-1, NF-κB and NF-IL-6 (Fig. 7B). It is known that these transcriptional factors are activated by LPS, TNF-α, IL-6 and IL-1β; therefore, the IDO gene could be up-regulated by LPS or these cytokines in a synergistic manner.

Recent studies have demonstrated that SB202190 suppresses the LPS-induced TNF-α protein and mRNA expression in macrophages (*29*, *30*). Further, an inhibitor of NF-κB also inhibits the production of TNF-α in monocyte/macrophage cells (*31*). Our study also demonstrates that SB202190 and SN50 partially inhibit TNF-α, IL-6 and IL-1β production in LPS-stimulated THP-1 cells (Fig. 6). These results suggest that the inhibitory effects of SB202190 and SN50 on IDO activity are partially mediated by the inhibition of the production of these cytokines. Possible mechanisms of IDO induction by LPS in monocyte or macrophage cells are summarized in Fig. 7A.

In conclusion, IDO is up-regulated by LPS and the combination of TNF-α, IL-6 or IL-1β in a synergistic manner in THP-1 cells. LPS induction of IDO is not related to the STAT1α or IRF-1 binding activities that are induced by IFN-γ, and NF-κB and/or the activity of the p38 MAPK pathway are involved.

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