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Curcumin Suppresses the Induction of Indoleamine 2,3-Dioxygenase by Blocking the Janus-activated Kinase-Protein Kinase Cδ-STAT1 Signaling Pathway in Interferon-γ-stimulated Murine Dendritic Cells*

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Indoleamine 2,3-dioxygenase (IDO) catalyzes the initial and rate-limiting step in the degradation of tryptophan and is strongly induced in interferon-γ (IFNγ)-stimulated dendritic cells (DCs). IDO has recently been established as a key enzyme in T-cell suppression-mediated immune tolerance to tumors. STAT1 phosphorylation appears to play an important role in the control of IDO expression by IFNγ, but the precise regulatory mechanism remains obscure. Here we present a novel mechanism of IFNγ-induced IDO expression in bone marrow-derived dendritic cells. In addition, we demonstrate that curcumin, an active component of turmeric, significantly inhibited the induction of IDO expression and activity by IFNγ. We found that curcumin suppressed STAT1 activation by directly inhibiting Janus-activated kinase 1/2 and protein kinase Cδ phosphorylation in bone marrow-derived DCs, suppressing the subsequent translocation and binding of STAT1 to the GAS element of the *IRF-1* promoter. Coincident with these inhibitory effects on IFNγ-induced IDO expression, curcumin reversed IDO-mediated suppression of T-cell responses. Our results, thus, suggest that down-regulation of IDO in DCs is an important immunomodulatory property of curcumin that may be exploited therapeutically in the control of cancers.

Dendritic cells (DCs)[3] are professional antigen-presenting cells that function as immune sentinels for the initiation of T-cell responses against microbial pathogens and tumors (1, 2). It is now well known that DCs not only induce immunity but are also important for the induction of T-cell tolerance. In particular, murine $CD11c^+$ DCs that coexpress the markers CD8α, B220, DX5, and DEC205 promote tolerance rather than immunity to specific antigens (3, 4). One of the mechanisms that might contribute to this tolerance in antigen-presenting cells involves the expression of the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO).

IDO catalyzes the initial and rate-limiting step in the catabolism of tryptophan along the kynurenine pathway. IDO has also recently been established as a key enzyme in T-cell suppression and the induction of immune tolerance (5–7). The expression of IDO by various cell types has broad immunological significance. In particular, in many tumors and tolerant antigen-presenting cells, IDO degrades tryptophan to kynurenine, leading to the depletion of tryptophan and resulting in the suppression of T-cell proliferation (8–10). Recent *in vivo* studies suggest that IDO-expressing DCs isolated from tumor-draining lymph nodes contribute to the progression of tumors by creating local immunosuppression (11–13).

The control of IDO transcription is complex and cell type-specific (6). A number of pathways, including the mitogen-activated protein kinase and noncanonical NF-κB signaling pathways as well as the Janus-activated kinase-signal transducer and activator of transcription (JAK-STAT) pathway, can modulate IDO expression in response to a variety of stimuli (14, 15). In macrophages and DCs, transcription of the *IDO* gene is strongly induced by IFNγ (6). In bone marrow-derived DCs (BMDCs), the precise regulatory mechanisms underlying IFNγ-induced IDO expression remain unclear.

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[3] The abbreviations used are: DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; PBS, phosphate-buffered saline; OVA, ovalbumin; ChIP, chromatin Immunoprecipitation; LPS, lipopolysaccharide; JAK, Janus-activated kinase; STAT, signal transducer and activator of transcription; BMDC, bone marrow (BM)-derived DC; IRF-1, IFN-regulatory factor-1; PKC, protein kinase C; Ab, antibodies; RT, reverse transcriptase; CFSE, carboxyfluorescein diacetate succinimidyl ester; GAS, gamma interferon activation sequences.

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IFNγ-induced gene expression has been closely linked to the JAK-STAT signaling pathway (16, 17). After IFNγ binds to its surface receptor, the receptor-associated JAK1 and JAK2 become activated and regulate the downstream phosphorylation of STAT1 on the tyrosine 701. Tyrosine phosphorylation of STAT results in the formation of STAT1-STAT1 homodimers, which translocate into the nucleus and bind to the distinct IFNγ-activated site (GAS) element in the promoters of IFNγ-activated genes, including IFN-regulatory factor-1 (IRF-1), to initiate gene transcription (17). In addition, STAT1 is also phosphorylated at serine 727 by protein kinase C δ (PKCδ), a phosphorylation event that is required for maximal induction of gene transcription (18). Both tyrosine and serine phosphorylation of STAT1 after the activation of JAKs and PKCδ are critically important in the transcription of IFNγ-associated genes (17). IFNγ-induced gene transcription in various cell types is also regulated by IRF-1, which transactivates multiple effector genes (20, 21). These regulatory effects are mediated by cooperative binding of STAT1 and IRF-1 to their cognate promoter sequence and are essential for IFNγ-induced gene transcription (19).

Curcumin (1,7-bis(4-hydrosy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), a phenolic natural product isolated from the rhizome of *Curcuma longa*, is the active component of turmeric. It exhibits anti-oxidant, anti-inflammatory, and anti-carcinogenic activities (22–25), implying that curcumin engages the immune system on multiple levels. On the basis of these multiple biological activities, curcumin has been suggested to have therapeutic potential in the treatment or prevention of several inflammatory diseases and cancer (26, 27).

The rationale for the present study stems from previous observations that several antioxidants inhibit IDO in INF-γ-activated macrophages through posttranslational or transcriptional regulation of IDO expression (28). In addition, we have previously reported that several antioxidants inhibit IDO expression in IFNγ-induced BMDCs (29–31). The present study was, thus, designed to investigate whether curcumin, acting as an antioxidant, could inhibit IDO expression and activity in IFNγ-induced murine BMDCs and, if so, to elucidate the underlying regulatory mechanism. In this report we demonstrate a novel biological activity of curcumin in IDO-mediated immune tolerance, showing that curcumin significantly inhibited the expression and enzymatic functions of IDO in IFNγ-stimulated murine BMDCs, thereby reversing IDO-mediated T-cell suppression. We further demonstrate that curcumin regulates IFNγ-induced IDO expression by targeting JAK1 and PKCδ signaling pathways.

EXPERIMENTAL PROCEDURES

Mice—Male 8–10-week-old C57BL/6 (H-2K^b and I-A^b) mice were purchased from the Korean Institute of Chemistry Technology (Daejeon, Korea). OT-1 T-cell receptor transgenic mice in the C57BL/6 background were purchased form The Jackson Laboratory (Bar Harbor, ME). They were bred using hemizygote males and wild-type females. To screen for transgenic offspring, 1 μl of blood was collected from the tail, immunostained with fluorescein isothiocyanate-labeled anti-Vα2 and Cy5-labeled anti-CD8 monoclonal antibodies (Abs), and analyzed using flow cytometry. Mice with nearly all of their CD8$^+$ T-cells stained positive for Vα2 were selected. The animals were housed in a specific pathogen-free environment within our animal facility and used in accordance with the institutional guidelines for animal care.

Reagents and Abs—Recombinant mouse (rm) granulocyte-macrophage colony-stimulating factor, rm interleukin-4, and rm INF-γ were purchased from R&D Systems (Minneapolis, MN). Curcumin with a purity of ≥80% and 1-methyl-DL-tryptophan was purchased from Sigma-Aldrich. InSolution^{TM} JAK Inhibitor I, rottlerin, Go6976, and safingol were purchased from Calbiochem. Fluorescein isothiocyanate- or phosphatidylethanolamine-conjugated monoclonal Abs used to detect the expression of CD11c (HL3), CD80 (16–10A1), and CD86 (GL1) were purchased from BD Pharmingen. To detect protein levels by Western blot, anti-phosphotyrosine-STAT1 (Tyr-701), anti-phospho-serine-STAT1 (Ser-757), and anti-STAT1 were purchased from Cell Signaling (Beverly, MA), and polyclonal anti-mouse IDO Ab was purchased from Alexis Biochemicals (San Diego, CA). Polyclonal rabbit anti-mouse Abs against α-tubulin, phospho-JAK1, phospho-JAK2, JAK2, phospho-PKCδ, and PKCδ were purchased from Santa Cruz Biotechnology, Inc.

Generation of BM-derived Murine DCs—DCs were generated from murine BM cells as described by Inaba *et al.* (32) with some modifications. BM was flushed from the tibiae and femurs of C57BL/6 and depleted of red cells with ammonium chloride. The cells were plated in 6-well culture plates (10^6 cells/ml; 3 ml/well) in Opti-MEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol, 10 mM HEPES, pH 7.4, 20 ng/ml recombinant mouse (rm) granulocyte-macrophage colony-stimulating factor and rm interleukin-4 at 37 °C, 5% CO_2. On day 3 of the culture, floating cells were gently removed, and fresh medium was added. On day 6 of the culture, nonadherent cells and loosely adherent proliferating DCs aggregates were harvested for analysis or stimulation. On day 6, 80% or more of the nonadherent cells expressed CD11c. To obtain highly purified populations for subsequent analyses, the DCs were labeled with bead-conjugated anti-CD11c monoclonal Ab (Miltenyi Biotec) and subjected to positive selection through paramagnetic columns (LS columns; Miltenyi Biotec) according to the manufacturer's instructions. The purity of the selected cell fraction was >90%.

IDO mRNA Analysis by Reverse Transcriptase (RT)-PCR—Total RNA from 5×10^6 cells was rapidly isolated using the TRIzol (Invitrogen) following the manufacturer's instructions. Total RNA (5 μg) was used for the synthesis of the first strand of cDNA. The oligonucleotides used for amplification of IDO were: 5′-GTACATCACCATGGCGTATG-3′ and 5′-GCTTTCGTCAAGTCTTCATTG-3′. Cycling conditions were 94 °C for 5 min (1 cycle); 94 °C for 30 s, 52 °C for 40 s, 72 °C for 40 s (32 cycles); 72 °C for 10 min (1 cycle). PCR products were fractionated on a 1.2% agarose-Tris-buffered EDTA gel containing ethidium bromide. For reference, we quantified the mouse glyceraldehyde-3-phosphate dehydrogenase gene.

Western Blot Analysis—Each cell lysate (30 μg of protein/lane) was subjected to electrophoresis on a 10% (w/v) polyac-

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rylamide gel and then transferred to a polyvinylidene difluoride microporous membrane. After the membrane was blocked, it was incubated with primary Ab and then incubated with horseradish peroxidase-conjugated anti-goat, anti-rabbit, or mouse-IgG secondary Ab. Finally, the membrane was developed with the ECL Western blotting detection reagent (Amersham Biosciences). Densitometric analysis was performed using an UN-SCAN-IT gel automated digitizing system (Scion, Frederick, MD).

Enzymatic Assay for IDO Activity—A strong correlation between the colorimetric assay and the high performance liquid chromatography analysis has been reported by Albert and coworkers. (33); we used the colorimetric assay for monitoring IDO activity. Briefly, 2×10^6 cells were disrupted by freeze/thaw cycles, the lysate (250 μl) was cleared by centrifugation, and an equal amount of 2× IDO buffer (100 mM PBS, pH 6.5, 40 mM ascorbate, 20 μM methylene blue, 200 μg/ml catalase, and 800 mM L-tryptophan; all reagents were purchased from Sigma-Aldrich) was added. After 30 min at 37 °C, the reaction was stopped with the addition of 100 μl of 30% trichloroacetic acid and further incubated for 30 min at 52 °C. After centrifugation, the supernatant was mixed with an equal amount of Ehrlich's reagent (0.8% *p*-dimethylaminobenzaldehyde in acetic acid). The color was allowed to develop for 10 min, and then the absorbance was read at 480 nm in a spectrophotometer. Serial dilutions of L-kynurenine were used as standards. A kynurenine standard curve was obtained by measuring the absorbance at 480 nm of kynurenine solutions at final concentrations of 0–100 μM (Fig. 1*C*, *inset*). One unit of IDO activity was defined as the amount of enzyme producing 1 nmol/h of kynurenine. The amount of protein in the samples was assayed according to the Bradford method using the Bio-Rad protein assay with bovine serum albumin as standard.

Mixed Lymphocyte Reaction—Transgenic ovalbumin (OVA)-specific $CD8^+$ T-cells were purified from bulk splenocytes via negative selection using a mouse $CD8^+$ T-cell kit (Miltenyi Biotec). Purity was assessed to be >93% by flow cytometry after staining with Cy5-conjugated anti-CD8 Ab. The lymphocytes were washed twice in PBS containing Ca^{2+} and Mg^{2+} and labeled by carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) as previously described (34). In brief, OT-1 T-cells were resuspended in 1 μM CFSE in PBS. After 8 min of shaking at 37 °C, cells were washed once in pure fetal bovine serum and twice in PBS with 10% fetal bovine serum. DCs (1×10^4 cells per well) generated from BM-derived monocytes of C57BL/6 were pretreated with 1-methyl-DL-tryptophan (600 μM) or curcumin (25 μM). Cells were then incubated for 18 h in the presence or absence of IFNγ (100 units/ml). DCs were pulsed or nonpulsed with 1 μM/ml OVA peptide (kindly provided by S. Y. Lee) at 37 °C for 1 h and then thoroughly washed before use. CFSE-labeled OT-1 T-cells were seeded in triplicate wells (1×10^5 per well) in U-bottomed, 96-well, microtiter culture plates (Nunc) together with DCs (1×10^4 per well). After 72 h of culturing, the cells were harvested and stained with Cy5-labeled anti-CD8 monoclonal Ab (to allow us to gate on the OT-1 T-cells) and analyzed by flow cytometry.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were carried out using a ChIP assay kit (Upstate Biotechnology) according to a modification of the manufacturer's instructions. 2×10^6 cells were fixed with 1% formaldehyde for 10 min at 37 °C to cross-link the protein-DNA complexes. Then they were harvested and washed twice with ice-cold PBS containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). For the remaining steps of the protein isolation, all buffers used to isolate the proteins contained phenylmethylsulfonyl fluoride and protease inhibitor mixture. Cells were added to SDS lysis buffer and incubated on ice for 10 min. Cell lysates were sonicated to shear the DNA to lengths between 200 and 1000 base pairs and then centrifuged at 14,000 rpm for 10 min at 4 °C. The sonicated cell supernatants were diluted 10-fold in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, and 167 mM NaCl) and precleared with a 27% suspension of protein A-agarose/salmon sperm DNA for 30 min at 4 °C with agitation. The supernatant was recovered after pelleting the agarose by centrifugation and then incubated with 5 μg of a rabbit-specific Ab against STAT1 overnight at 4 °C. The Ab-protein-DNA complexes were collected by adding protein A-agarose/salmon sperm DNA for 1 h at 4 °C with agitation. Immunoprecipitated Ab-protein-DNA complexes were washed 3 times with wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM NaCl) followed by three additional washes in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Chromatin complexes were eluted with 250 μl of freshly prepared extraction buffer (1% SDS, 0.1 M $NaHCO_3$). To reverse cross-links, 5 M NaCl was added to each combined elute to a final concentration of 0.3 M, then the solution was heated to 65 °C for 5 h. Proteins were digested with 100 μg/ml proteinase K for 1 h at 45 °C, and DNA was extracted with a QIAquick PCR purification kit (Qiagen). Precipitated DNA fragments were amplified by PCR. The sequences of *IRF-1* promoter primer are as follows: IRF-1 forward primer 5′-CTTTCCAACACAGGCAAG-3′, IRF-1 reverse primer 5′-ACTGTGAAAGCACGTAC-3′.

Statistics—Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the mean ± S.E. Analysis of variance was used to compare experimental groups to control values, whereas comparisons between multiple groups were done using Tukey's multiple comparison test. Statistical significance was determined a p value less than 0.05.

RESULTS

Curcumin Inhibits the Expression and Activity of IDO in IFNγ-stimulated BMDCs—To investigate the effect of curcumin on IFNγ-induced IDO expression, we preincubated BMDCs with or without curcumin and then stimulated cells with IFNγ. Using RT-PCR, we observed that IFNγ induced a significant increase in the transcription of the IDO gene in BMDCs, an increase that was inhibited in a dose-dependent manner by curcumin (Fig. 1*A*). Western blot analyses confirmed that curcumin also inhibited IFNγ-induced IDO expression at the protein level (Fig. 1*B*). We have previously shown that curcumin has no remarkable effect on cell viability at cur-

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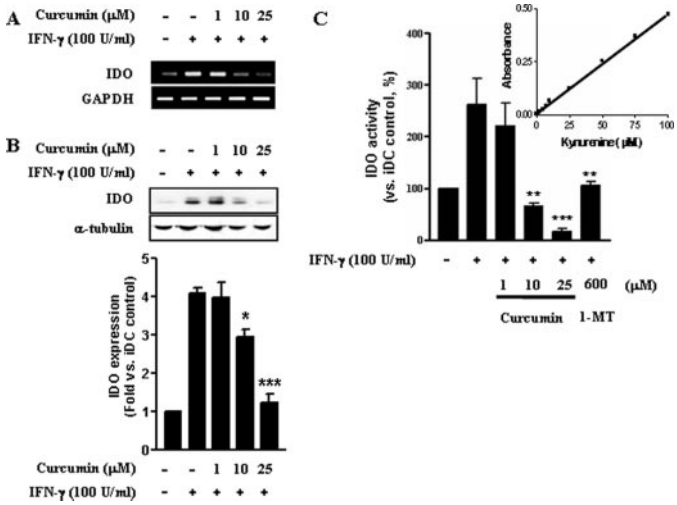

FIGURE 1. **Effect of curcumin on IDO expression and activity in IFNγ-stimulated murine BMDCs.** BMDCs were incubated for 1 h in the absence or presence of various concentrations (1–25 μM) of curcumin and further incubated with IFNγ (100 units/ml) for 18 h. *A*, the relative levels of IDO mRNA were measured using RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a housekeeping gene, is shown as a loading control. *B*, protein levels in cell extract were detected by Western blotting using a polyclonal anti-mouse IDO Ab. Total protein in the sample was equalized before loading the gel. α-Tubulin was used as a loading control. *Upper panel*, representative blots of three independent experiments. *Lower panel*, densitometric analysis of the immunoblots presented in the *upper panel*. *C*, a colorimetric assay was used to monitor IDO activity, determined as kynurenine formation, as described under "Experimental Procedures." Values, presented as percent of control (untreated immature DCs), are the mean ± S.E. of three experiments. Significant decreases compared with BMDCs stimulated with IFNγ are indicated by *asterisks*; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

cumin concentrations ≤50 μM (35), indicating that these inhibitory effects on IDO expression were not due to nonspecific cytotoxic consequences of curcumin exposure.

To determine whether reductions in IDO expression were accompanied by a decrease in IDO activity in IFNγ-activated BMDCs, we assayed cell extracts for the presence of kynurenine, a byproduct of tryptophan. As shown in Fig. 1*C*, a non-toxic concentration of curcumin (25 μM) induced a dramatic, dose-dependent inhibition of IFNγ-induced IDO activity.

JAK1/2 and PKCδ Regulate Tyrosine and Serine Phosphorylation of STAT1 in IFNγ-stimulated BMDCs—Because curcumin strongly suppressed the induction of IDO expression and activity in IFNγ-stimulated BMDCs, we next sought to examine the precise mechanisms involved in the induction and inhibition of IDO expression by IFNγ and curcumin, respectively. We first investigated whether phosphorylation of tyrosine and serine residues of STAT1 was regulated by JAK1/2 and PKCδ in IFNγ-stimulated murine BMDCs. JAK1/2, PKCδ, and STAT1 were rapidly activated in response to IFNγ, reaching a plateau 30 min after IFNγ stimulation (Fig. 2*A*). Pretreatment with JAK-specific inhibitor was completely suppressed tyrosine phosphorylation of STAT1 in IFNγ-stimulated BMDCs, whereas serine phosphorylation of STAT1 and phosphorylation of PKCδ was not changed (Fig. 2*B*). Next, to determine whether phosphorylation of STAT1 in response to IFNγ was dependent on PKCδ, we preincubated BMDCs with the PKCδ-specific inhibitor, rottlerin, for 30 min before stimulating with IFNγ for 30 min. Rottlerin abrogated IFNγ-induced serine phosphorylation of STAT1 without decreasing STAT1 tyrosine phosphorylation (Fig. 2*C*).

PKC comprises a family of serine/threonine kinases that transducer-signals and regulate the expression of genes involved in cell proliferation, differentiation, apoptosis (36). To further explore the specificity of PKCδ in regulating IFNγ-induced IDO expression, we determined whether other PKC isotypes could regulate serine phosphorylation of STAT1 in response to IFNγ. Go6976, which inhibits PKC-α, PKC-β, and PKC-μ, and safingol, which specifically inhibits PKC-α, were examined for their ability to inhibit STAT1 serine phosphorylation. BMDCs were preincubated with effective doses of the PKC inhibitors for 30 min and then treated with IFNγ for 30 min. As shown in Fig. 2*D*, these PKC inhibitors, which do not inhibit PKCδ, did not affect the IFNγ-dependent serine phosphorylation of STAT1.

Activation of Both JAK1/2 and PKCδ Is Essential for IDO Expression in IFNγ-stimulated BMDCs—To characterize the direct effect of inactivating JAK1/2 and PKCδ on IDO induction by IFNγ, we investigated IDO expression in IFNγ-stimulated BMDCs treated with either the JAK1/2- or PKCδ specific inhibitor. Using RT-PCR and Western blot analysis, we observed that IFNγ-induced IDO expression was completely suppressed by the JAK inhibitor (Fig. 3*A*). Similarly, as shown in Fig. 3*B*, treatment with the PKCδ inhibitor, rottlerin, reduced IDO expression in a dose-dependent manner in IFNγ-stimulated BMDCs. In contrast, inhibitors of other PKC isoforms had little effect on IDO expression in IFNγ-stimulated BMDCs (Fig. 3*C*). To exclude the possibility that the effect of rottlerin on IFNγ-induced IDO expression were nonspecific, we used PKCδ-specific small interfering RNA to silence the PKCδ gene in BMDCs. Introduction of PKCδ-specific small interfering RNA significantly reduced IDO expression and PKCδ phosphorylation in response to IFNγ (Fig. 3*D*).

Curcumin Inhibits STAT1 Activation by Suppressing IFNγ-induced of JAK1/2 and PKCδ Phosphorylation—The dependence of IFNγ-induced IDO expression on JAK1/2 and PKCδ phosphorylation suggests that curcumin may block IFNγ effects by inhibiting these downstream events. To investigate the effect of curcumin on the phosphorylation of JAK1/2 and PKCδ, we preincubated BMDCs with varying doses of curcumin for 30 min and then stimulated cells with IFNγ for 30 min. Curcumin treatment dramatically reduced the phosphorylation of JAK1/2 and PKCδ in IFNγ-stimulated BMDCs (Fig. 4*A*).

Next, we examined whether the inhibitory effect of curcumin on JAK1/2 and PKCδ activation suppressed the subsequent phosphorylation of STAT1. As expected, curcumin significantly reduced STAT1 phosphorylation on both serine and tyrosine residues (Fig. 4*B*).

Curcumin Inhibits the Binding of Phosphorylated STAT1 to the GAS Site of the IRF-1 Promoter in IFNγ-stimulated BMDCs—The binding of phosphorylated STAT1 to the GAS site of the IRF-1 promoter is essential for the induction of IRF-1 gene transcription by IFNγ (37). Moreover, STAT1 and IRF-1 cooperatively mediate the induction of IDO protein by IFNγ (16). To determine whether inhibiting JAK1/2 or PKCδ impaired the binding capacity of IFNγ-activated STAT1 to the GAS site of the IRF-1 promoter and blunted subsequent IRF-1

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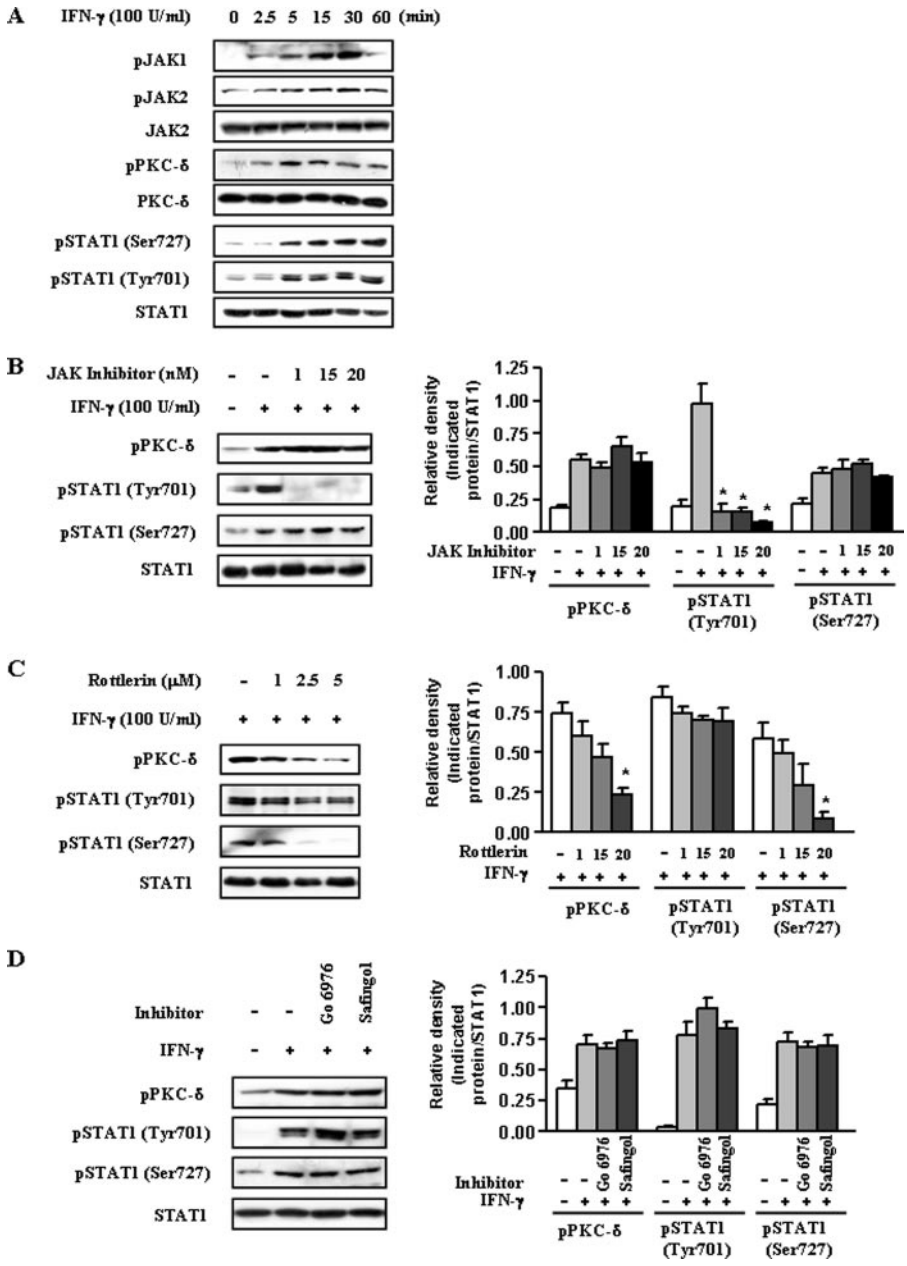

FIGURE 2. **JAK1/2 and PKCδ phosphorylation is associated with full activation of STAT1.** *A*, after treatment with IFNγ (100 units/ml), cells were harvested at the indicated times. *B–D*, before incubating with 100 units/ml IFNγ for 30 min, BMDCs were treated for 30 min with the JAK inhibitor, rottlerin, Go6976 (20 nM), or safingol (40 μM). Cell lysates were immunoblotted with anti-phospho (*p*)-PKCδ, anti-PKCδ, anti-phosphotyrosine, anti-phosphoserine STAT1, and anti-STAT1 Abs. Each blot is representative of three independent experiments. The *bar graphs* show densitometric analysis of immunoblots; the phosphorylation level for each protein represents the intensity of each band relative to STAT1 levels. Data are the means ± S.E. of three separate experiments. *, $p < 0.001$ compared with LPS-treated BMDCs.

expression, BMDCs were preincubated with a JAK- or PKCδ-specific inhibitor before IFNγ treatment. In the ChIP assay using STAT1-specific Ab and Western blot analyses, we observed that inhibiting JAK1/2 or PKCδ activation severely blunted or abolished the binding of IFNγ-activated STAT1 to the GAS sequence (Fig. 5*A*) and reduced subsequent IRF-1 expression (Fig. 5*B*). This latter effect was more pronounced in the presence of both inhibitors.

We next investigated whether curcumin treatment attenuated STAT1 binding to the *IRF-1* promoter in IFNγ-stimulated BMDCs using the ChIP assay. Cells were preincubated with various concentrations of curcumin for 1 h before IFNγ treatment. Chromatin was isolated and immunoprecipitated with a STAT1-specific Ab, and then the *IRF-1* promoter region was amplified by PCR. Curcumin treatment significantly blocked STAT1 binding to the GAS elements on the promoter region of the *IRF-1* gene (Fig. 6*A*, *upper panel*). Similar effects of curcumin were observed in ChIP assays using an RNA polymerase II-specific Ab (Fig. 6*A*, *lower panel*). In addition, Western blots showed that IRF-1 expression, which was strongly induced in the IFNγ-stimulated BMDCs, was profoundly inhibited after curcumin-mediated suppression of STAT1 binding to the GAS element (Fig. 6*B*).

Curcumin Restores IDO-induced T-cell Suppression—Finally, we sought to determine whether curcumin treatment could reverse IDO-mediated suppression of T-cell proliferation. Paradoxically, in a previous study of LPS-induced DC maturation, we found that curcumin caused a suppression of the T-cell stimulatory function by down-regulating the expression of co-stimulatory molecules (35). This observation prompted us to analyze the expression levels of co-stimulatory molecules in IFNγ-stimulated BMDC. Consistent with our previous report, LPS was sufficient to induce the expression of CD80 and CD86 molecules on $CD11c^+$ cells on day 7, whereas IFNγ did not (Fig. 7*A*). Although curcumin modulates the expression of CD80 and CD86 and major histocompatibility complex (MHC) class II molecules in LPS-stimulated BMDCs, it has little inhibitory effect on the expression of MHC class I molecules (35). To precisely characterize the effect of curcumin on IDO-mediated T-cell suppression separate from the effect of curcumin on the expression of co-stimulatory molecules, we established mixed lymphocyte reaction system using OT-1 T-cell receptor (TCR) transgenic $CD8^+$ T-cells, which express a TCR specific for the major histocompatibility complex I-restricted OVA peptide 257–264 antigen in DCs (50). Transgenic OVA-specific $CD8^+$ T-cells co-cultured with IFNγ-stimulated DCs proliferated to a significantly lesser

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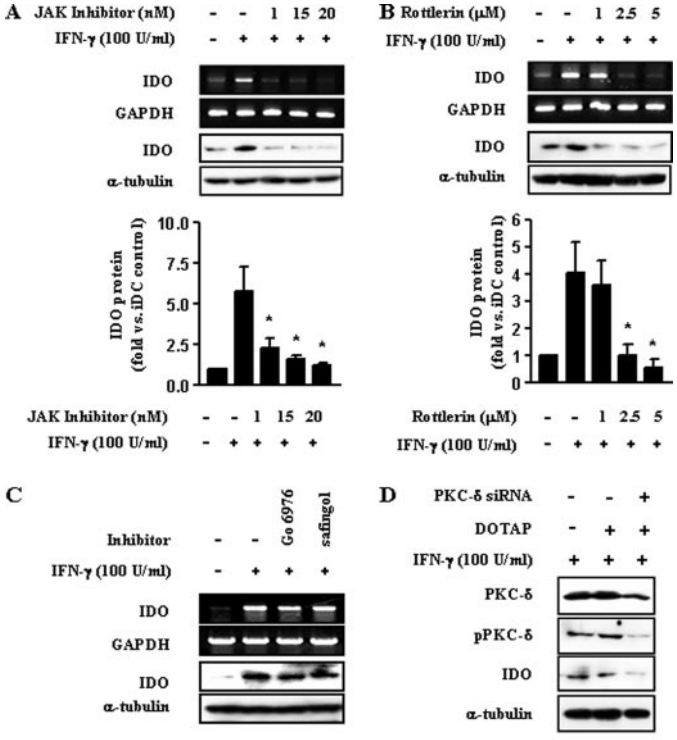

FIGURE 3. **JAK1/2 and PKCδ phosphorylation is important for IDO expression in IFNγ-stimulated murine BMDCs.** *A* and *B*, BMDCs were incubated in the presence or absence of either a specific inhibitor of JAK1/2- or PKCδ (rottlerin) for 30 min at the indicated concentration before stimulating with IFNγ (100 units/ml). Cells were then immediately lysed with TRIzol, and IDO and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA expression was detected by RT-PCR. Equal amounts of total cell lysates were also immunoblotted with anti-IDO Abs. *Lower panel*, densitometric analysis of each Western blot. Values are the mean ± S.E. of three experiments. *, $p < 0.05$ compared with LPS-treated BMDCs. *C*, before incubating with 100 units/ml IFNγ for 18 h, BMDCs were treated for 30 min with 20 nM Go6976 or 40 μM safingol. RT-PCR and immunoblotting were performed as described in *A* and *B*, above. *D*, effects of specific PKCδ gene silencing on IFNγ-stimulated BMDCs. The cells were treated with or without a small interfering RNA targeting the PKCδ gene for 24 h. Control cells were treated with DOTAP alone. Cells lysates were immunoblotted with anti-PKCδ, anti-phospho PKCδ, anti-IDO, and anti-α-tubulin Abs.

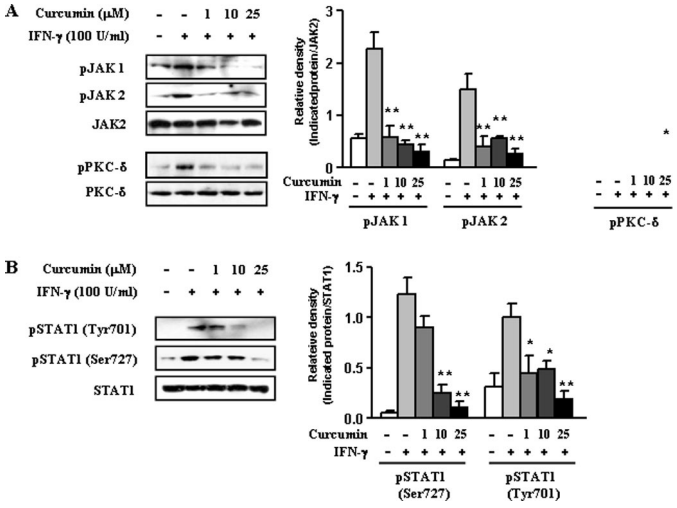

FIGURE 4. **Curcumin inhibits phosphorylation (*p*) of JAK1/2 and PKCδ in IFNγ-stimulated BMDCs and leads to the suppression of IFNγ-induced STAT1 phosphorylation.** *A* and *B*, BMDCs were pretreated with the indicated curcumin concentrations for 30 min and harvested after incubating with IFNγ for 30 min. Whole-cell lysates were immunoblotted using Abs against the proteins indicated at the *left* of the figure. To verify that the total level of each protein did not change, the blots were stripped and reprobed with anti-STAT1, anti-JAK2, and anti PKCδ Abs. *Bar graphs* show densitometric analysis of immunoblot; the phosphorylation level for each protein represents the intensity of each band relative to JAK2, PKCδ, or STAT1 levels). Data are the means ± S.E. of three separate experiments. $p < 0.05$ (*) and $p < 0.001$ (**) compared with LPS-treated BMDCs.

extent than did the same T-cells co-cultured with untreated control DCs. This suppressive effect of IFNγ stimulation was completely reversed by treatment of DCs with 1-methyl-DL-tryptophan, a well characterized competitive inhibitor of IDO. Similarly, the proliferation potential of OVA-specific OT-1 cells was restored by co-culturing with DCs pretreated with curcumin (25 μM) before IFNγ stimulation (Fig. 7*B*).

DISCUSSION

We have previously reported that the anti-inflammatory effect of curcumin can be attributed to the inhibition of mitogen-activated protein kinase and NF-κB, mediators involved in cytokine signaling and inflammation (35). Here, we demonstrate an additional biological property of curcumin showing that curcumin also regulates IFNγ-induced IDO, a key enzyme in T-cell suppression and the induction of immune tolerance. Consistent with this activity, we found that curcumin reversed IDO-mediated T-cell suppression, restoring normal proliferative potential to $CD8^+$ T-cells. The main purpose of this study was to determine the effect and mechanism of action of curcumin on IDO-mediated immune regulation. Although it is well established that signaling via the JAK-STAT pathway plays a critical role in mediating the biological effects of IFNγ, specific information on the mechanisms by which IFNγ induces IDO expression in murine BMDCs has been lacking. We observed that blocking JAK1 and JAK2 activation inhibited the STAT1 tyrosine phosphorylation and induction of *IDO* transcription that normally occur upon exposure to IFNγ. Moreover, down-regulation of PKCδ either with a chemical inhibitor or through small interfering RNA-mediated silencing suppressed IDO expression by inhibiting serine, but not tyrosine phosphorylation of STAT1 in IFNγ-stimulated DCs. These results indicate that tyrosine and serine phosphorylation of STAT1 are specifically regulated by JAK and PKCδ, respectively, and demonstrate that full activation of STAT1 is crucial for IFNγ-induced IDO expression in murine BMDCs.

The initiation of IDO gene transcription is a complex process that is initiated by the expression of a key transcriptional factor, IRF-1, upon IFNγ stimulation (16). The IRF-1 gene promoter contains a GAS sequence, which binds STAT1 homodimers; IFNγ-mediated IRF-1 expression is driven primarily by phosphorylated STAT1 homodimers that bind to this element. In addition, fully activated STAT1 indirectly binds the GAS element of the target gene promoter through an association with IRF-1 (Fig. 8) (41, 42). Using ChIP assays, we showed that inhibition of STAT1 tyrosine phosphorylation impairs the ability of STAT1 to bind to the element region of the IRF-1 promoter that contains the GAS element. This defect in GAS element binding that accompanies inhibition of STAT1 phosphorylation reduces the subsequent expression of IRF-1 as well as IDO. These data indicate that IRF-1 plays a pivotal role in the IFNγ-induced IDO expression.

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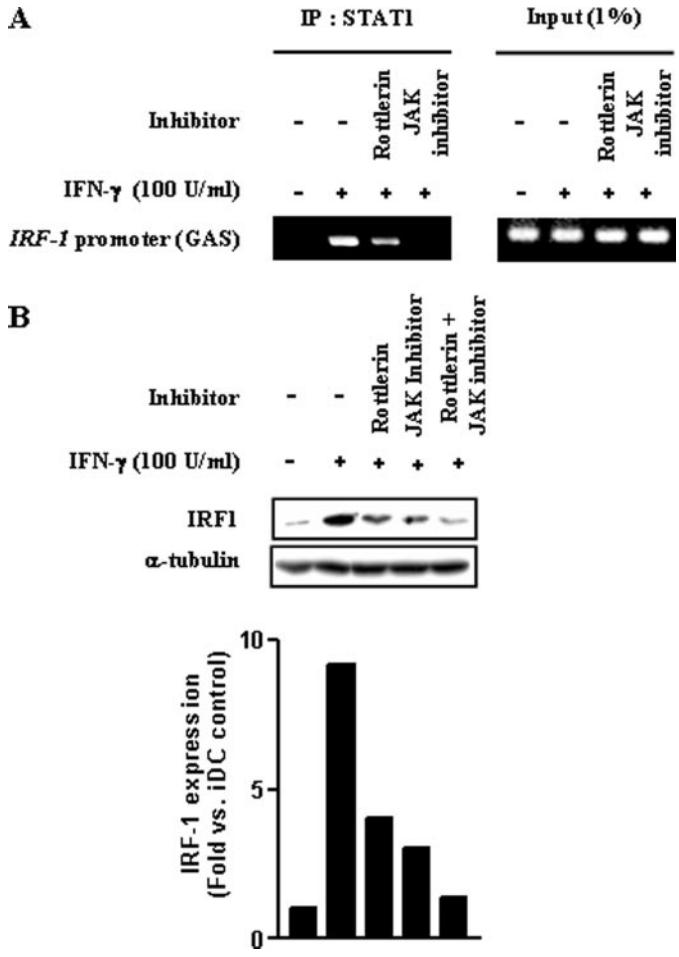

FIGURE 5. **Inhibition of JAK1/2 or PKCδ impairs the binding of phosphorylated STAT1 to the GAS site of the *IRF-1* promoter and inhibits IRF-1 expression in IFNγ-stimulated murine BMDCs.** *A*, BMDCs were pretreated with either a JAK inhibitor (20 nM) or PKCδ inhibitor (rottlerin, 5 μM) for 1 h and then stimulated with IFNγ (100 units/ml) for 1 h. A ChIP assay was performed after immunoprecipitation (*IP*) of chromatin with anti-STAT1 Ab. Immunoprecipitated chromatin and 1% of input chromatin used for each immunoprecipitation were subjected to PCR with primers specific for the *IRF-1* promoter. *B*, BMDCs were pretreated with the JAK inhibitor (20 nM), rottlerin (5 μM), or both for 1 h and stimulated with IFNγ for 2 h. Cell lysates were analyzed by Western blotting using Abs against IRF-1 and α-tubulin. Relative levels of IRF-1 expression normalized to α-tubulin were determined by densitometric scanning of IRF-1 and α-tubulin bands. Data shown are representative two independent experiments.

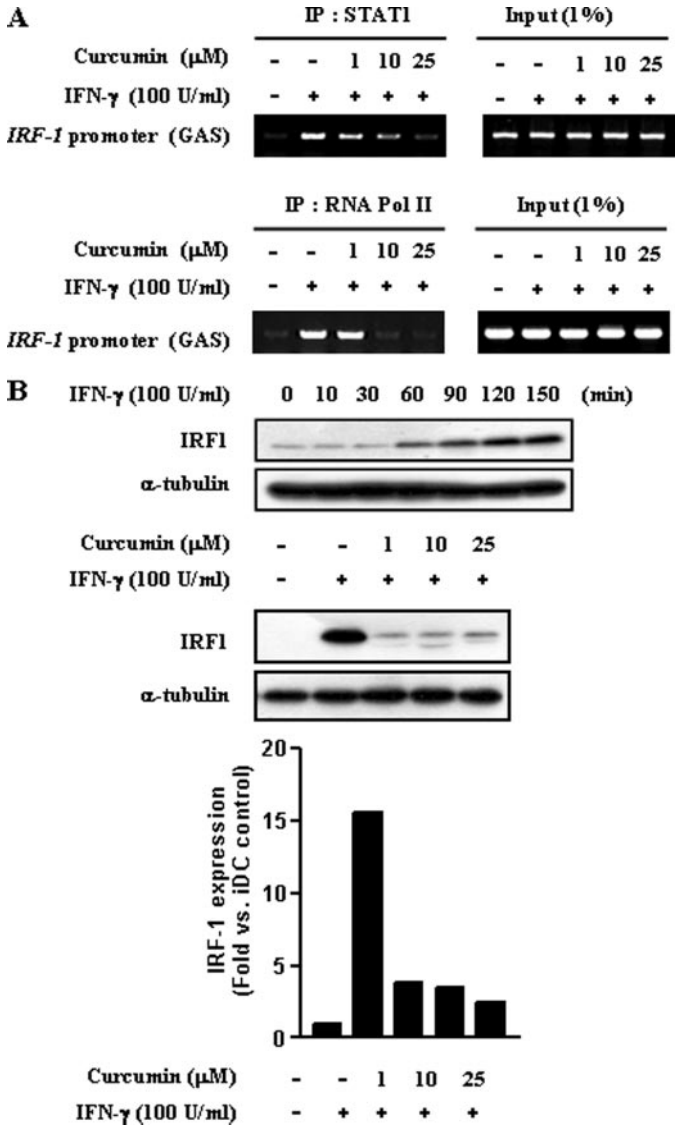

FIGURE 6. **Curcumin suppresses the binding of activated STAT1 to the GAS site of the *IRF-1* promoter and inhibits IRF-1 expression in IFNγ-stimulated BMDCs.** *A*, BMDCs were pretreated with curcumin 1, 10, or 25 μm for 1 h and stimulated with IFNγ 100 units/ml for 1 h. ChIP assays were performed after immunoprecipitation (*IP*) of chromatin with anti-STAT1 (*upper panel*) Ab or anti-RNA polymerase II (*lower panel*) Abs. Immunoprecipitated chromatin and 1% of input chromatin used for each immunoprecipitation were subjected to PCR with primers specific for the *IRF-1* promoter. *B*, BMDCs were stimulated with 100 units/ml IFNγ and harvested at the indicated time periods (*upper panel*). Cells were pretreated with the indicated curcumin concentrations for 1 h and stimulated with IFNγ for 2 h (*lower panel*). Cell lysates were analyzed by Western blotting using Abs against IRF-1 and α-tubulin. Relative levels of IRF-1 expression (normalized to α-tubulin) were determined by densitometric scanning of IRF-1 and α-tubulin bands. Data shown represent of two independent experiments.

In light of the mechanism of IFNγ-induced IDO expression described above, we are able to understand how curcumin inhibits the expression of IDO in murine BMDCs treated with IFNγ. Thus, we conclude that the inhibition of IDO by curcumin is caused by suppression of the JAK-PKCδ-STAT1 signaling pathway. Indeed, curcumin inhibits JAK1/2 and PKCδ activation, thereby leading to the inhibition of STAT1 phosphorylation (Fig. 4). This decrease in STAT1 phosphorylation attenuates the capacity of the STAT1 homodimer to bind to the *IRF-1* promoter and reduces IRF-1 expression in IFNγ-stimulated BMDCs. A decrease in IRF-1 expression reduces STAT1 and IRF-1 binding to the GAS and interferon-stimulated response element sequence of the IDO gene promoter region and subsequently impairs the initiation of IDO gene transcription. Thus, curcumin acts at multiple points in the signaling cascade that underlies IFNγ-induced IDO expression (Fig. 8).

Recently, it has been shown that IDO-competent DCs are rendered highly tolerogenic by IFNγ, which activates IDO and initiates the immunosuppressive pathway of tryptophan catabolism (11, 43, 44). There is increasing evidence to indicate that murine splenic $CD8\alpha^+$ DCs can mediate apoptosis of T-cell clones; this effect can be reversed by the addition of an IDO inhibitor (45–47). Likewise, in our experiment using OT-1 T-cell receptor transgenic $CD8^+$ T-cells, we clearly showed

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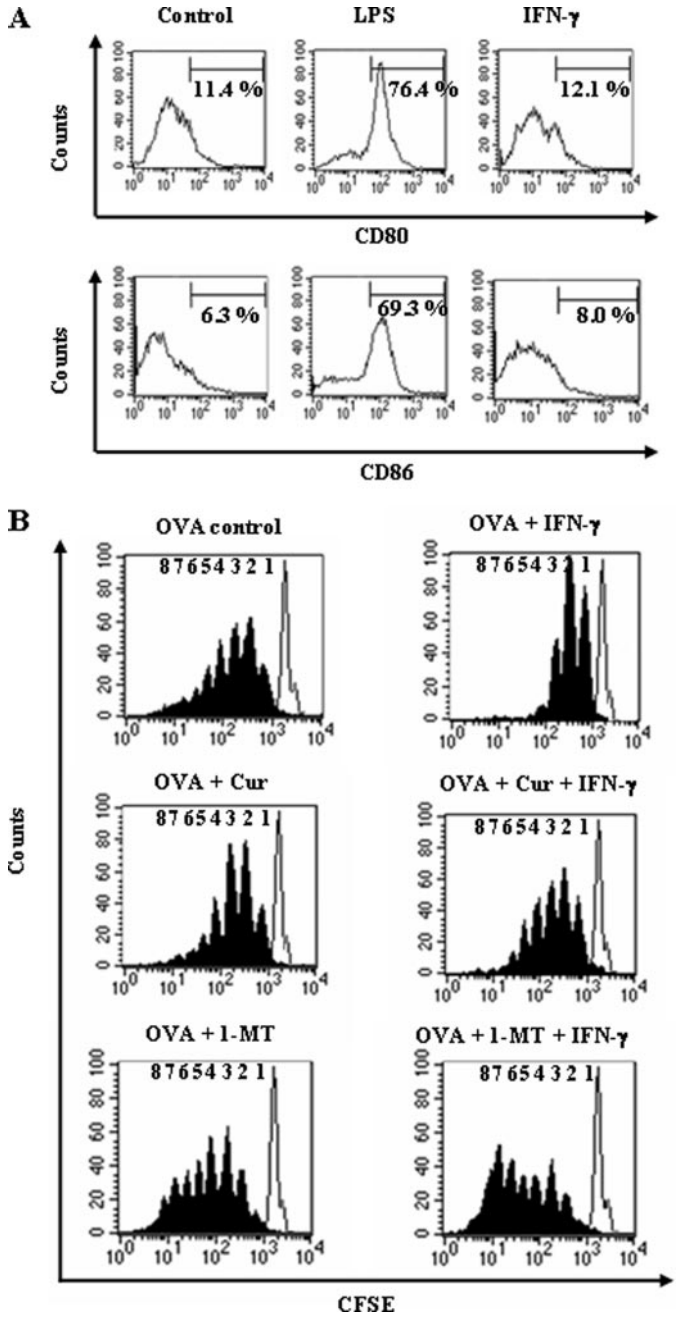

FIGURE 7. **Effect of curcumin on the T-cell suppression induced by IDO *in vitro*.** *A*, BMDCs were generated as described under "Experimental Procedures." On day 6 the cells were incubated with LPS (200 ng/ml) or IFNγ (200 units/ml) for 24 h. On day 7 cells were harvested and analyzed by two-color flow cytometry. The results shown are representative of three independent experiment. *B*, transgenic OVA-specific $CD8^+$ T-cells were isolated and stained with 1 μM CFSE and cocultured for 96 h with DCs loaded with OVA peptide (1 μg/ml) as described under "Experimental Procedures." T-cell proliferation was assessed by flow cytometric analysis of CFSE dilution. Flow histograms of CFSE fluorescence for $CD8^+$ gated T-cells after T-cell stimulation with OVA peptide-pulsed DCs (*dark histogram*) or non-pulsed DCs (*light histogram*) are shown. Numbers indicate evidence of cell division.

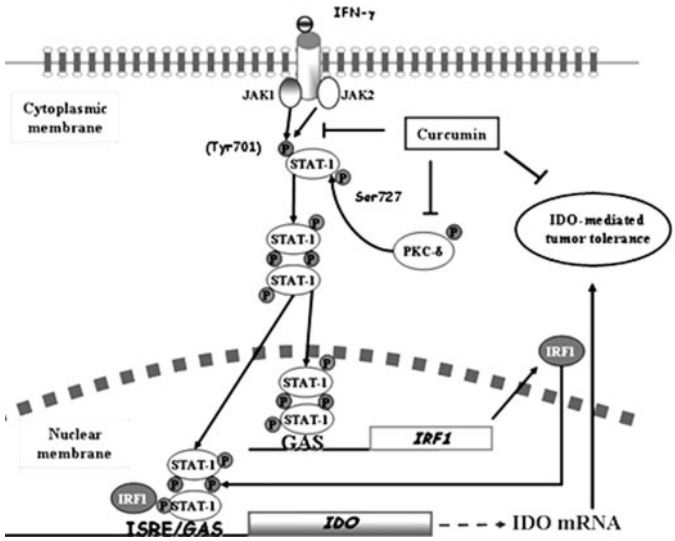

FIGURE 8. **Curcumin regulation of IFNγ-induced IDO expression in murine BMDCs.** Binding of IFNγ to its receptor induces JAK1/2 phosphorylation and PKCδ phosphorylation. Phosphorylation of STAT1 on both tyrosine and serine residues after the activation of JAKs and PKCδ is required for STAT1 dimerization and translocation into the nucleus. Activated STAT1 binds to the GAS site of the IDO and *IRF-1* promoter and induces IRF-1 expression. IRF-1 binds to the interferon-stimulated response element (*ISRE*) of the IDO promoter and activates it in cooperation with STAT1 IFNγ induces activation of JAK1/2, PKCδ, and STAT1. Inhibition of the JAK-PKCδ-STAT1 pathway by curcumin inhibits IDO expression and, ultimately, suppresses IDO-mediated tumor tolerance.

that curcumin reversed the suppression of $CD8^+$ T-cell proliferation that was caused by IFNγ-induced IDO expression. We propose that the beneficial effect of curcumin in IDO-mediated T-cell suppression occurs primarily by interfering with the cascade of events that ultimately leads to IFNγ-induced IDO expression. In a previous study on LPS-induced DC maturation, we reported that curcumin down-regulates the expression of a co-stimulatory molecule and thereby suppresses the T-cell stimulatory function of these DCs (35). Differences in the T-cell proliferative effect of curcumin between this previous report and the present study might be due to differences in the extent to which LPS and IFNγ stimulate DCs and induced IDO expression. In fact, the effect of curcumin on the immunological responses of murine DCs may be stimulus-specific. LPS was more potent in inducing co-stimulatory molecules than was IFNγ; conversely, IFNγ was more potent in inducing IDO than was LPS. A previous study suggested that IFNγ, unlike LPS, can directly down-regulate the antigen-presentation ability of DC (48). As shown here (Fig. 7*A*), LPS, but not IFNγ, can induce the expression of the co-stimulatory molecules, CD80 and CD86. That these differences between LPS and IFNγ extend to their effects on IDO induction is demonstrated by our previous studies, which showed that IFNγ, as a principal IDO inducer, increases the transcription of the *IDO* gene to levels that are more than 3-fold higher than after LPS stimulation (14). In addition, results of IFNγ neutralization and IDO inhibition experiments indicate that IFNγ-induced IDO expression is closely linked to DC-mediated T-cell suppression (49). These results indicate that IFNγ-stimulated DCs have an anti-inflammatory role that reflects the induction of IDO. Thus, although curcumin suppresses the expression of CD80, CD86, and major histocompatibility complex class II molecules in LPS-stimulated BMDCs (35), the restoration of normal T-cell proliferation by curcumin pretreatment may have more to do with efficient blockade of IDO than co-stimulatory capacity, as shown in Fig. 7*B*.

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The molecular mechanisms underlying immunologic tolerance, which allows a tumor to escape from host immune surveillance, have been identified and are currently the subject of active research (38). IDO plays an important role in tumor immunology and is a primary molecular target for cancer immunotherapy. Accumulating evidence suggests that strategies enhances the efficacy of other immunotherapies (39, 40). As such, the identification of more potent and effective IDO inhibitors may be a key to the development of more effective tumor immunotherapy regimens. Our data clearly offer new insights into the biochemical mechanisms that govern IDO expression in response to IFNγ and the immune-modulatory activities of curcumin. They also provide a framework for designing better DC- and biochemical-based immunotherapy trials in the future.

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Curcumin Suppresses the Induction of Indoleamine 2,3-Dioxygenase by Blocking the Janus-activated Kinase-Protein Kinase C δ-STAT1 Signaling Pathway in Interferon- γ-stimulated Murine Dendritic Cells

Young-Il Jeong, Sang Woo Kim, In Duk Jung, Jun Sik Lee, Jeong Hyun Chang, Chang-Min Lee, Sung Hak Chun, Man-Soo Yoon, Geun Tae Kim, Seok Woo Ryu, Jong-Suk Kim, Yong Kyoo Shin, Won Suk Lee, Hwa Kyoung Shin, Jae-Dong Lee and Yeong-Min Park

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