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Rosmarinic acid inhibits indoleamine 2,3-dioxygenase expression in murine dendritic cells

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ABSTRACT

Indoleamine 2,3-dioxygenase (IDO), a key enzyme that catalyses the initial and rate-limiting step in the degradation of the tryptophan, is simultaneously expressed in murine dendritic cells and macrophages stimulated with interferon-γ (IFN-γ). In the present study, we investigated whether rosmarinic acid (RA), which is suggested to exhibit anti-oxidant and anti-cyclooxygenase properties, could suppress the functional expression of IDO in murine bone marrow-derived dendritic cells (BMDCs) stimulated with IFN-γ. Treatment with RA reduced intracellular expression of IDO both in IFN-γ-activated BMDCs *in vitro* and in $CD11c^+CD8\alpha^+$ DCs *in vivo* tumor-bearing mice model. Consequently, we obtained evidence that RA suppresses the functional activity of IDO and blocks the IDO-dependent T cell suppression. In IFN-γ-mediated induction of IDO transcription, activation of the signal transducer and activator of transcription 1 (STAT1) is important to be express IDO in IFN-γ-stimulated BMDCs. In this study, we demonstrated that the RA could also suppress IFN-γ-induced STAT1 activation. These novel findings provide a new insight into that RA as a pharmacological and transcriptional inhibitor of IDO is worthy of clinical application as well as further investigation for IDO regulation.

1. Introduction

Dendritic cells (DCs) are professional antigen presenting cells (APCs) playing key roles in the immune sentinels as initiators of T cell responses against microbial pathogens and tumors [1,2]. Immature DCs (iDCs) capture and process exogenous agents in peripheral tissues and begin to mature. The maturing DCs migrate to lymphoid organs, where they stimulate naïve T cells through the signals of both major histocompatability complex (MHC) molecules presenting antigen-peptides and co-stimulatory molecules [3,4]. However, it is now well appreciated that DCs not only induce immunity but also play a major role during the induction of T cell tolerance, particularly in cancer patients [5]. Accumulating evidence indicates that $CD8\alpha^+$ DCs can also induce tolerance, rather than immune activation, to the antigens they present [6]. One mechanism that might

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contribute to this tolerance is the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO).

IDO is an enzyme catalyzing the initial and rate-limiting step in the catabolism of tryptophan along the kynurenine pathway [7], and has recently been established as a key enzyme in T cell suppression and induction of immune tolerance to tumor [8,9]. The biological role of IDO in the immune system has been implicated that inhibition of T cell proliferation by tryptophan catabolism protects the fetus from maternal responses. The expression, however, of IDO by different cell types could have broader immunological significance in T cell homeostasis and tolerance. Previous study has been shown that human DC expressing significant IDO activity can mediate inhibition of T cell proliferation through tryptophan degradation [10]. The overall immunosuppressive effects of IDO are not entirely known, but may depend on the type of IDO-expressing cell or its subset [11]. More recently, splenic DCs in the CD8α^+ subset can mediated IDO-dependent apoptosis of T cells [5,12,13]. In fact, IDO activity in the CD8α^+ subset DCs is increased under pathological conditions including tumor development [14]. Especially, in many tumors and on tolerzing APCs, its expression is induced [15]. Recent report suggests that IDO-expressing DCs isolated from tumor-draining lymph nodes contributes to the progression of tumors by creating profound local immunosuppression and T cell anergy *in vivo* [8,9].

The tumor microenvironment is characterized by high expression of immunosuppressive factors including COX-2 and its product, PGE_2 [16,17]. PGE_2 is a known inducer of IDO expression in APCs [18,19]. Previous work describing that specific inhibiting COX-2 reduces IDO expression within the tumor microenvironment [20].

The control of IDO transcription is complex and cell-type specific, and various signaling pathways might modulate IDO expression by specific cell types. The expression of the IDO gene is induced strongly by IFN-γ in macrophages or DCs. It has been demonstrated that JAK/STAT1 signaling pathway and IFN-regulatory factor-1 (IRF-1) mediate cooperatively the induction of IDO expression by IFN-γ [21–24]. In addition, in terms of the transcriptional induction of IDO gene by IFN-γ, previous studies support that the induction of IFN-γ-inducible, dsRNA-activated serine/threonine protein kinase (PKR) may function through STAT transcription factors [21].

Rosmarinic acid (α-ocaffeoyl-3,4-dihydroxyphenyl-lactic acid; RA), as a secondary metabolite of herbs (Fig. 1), has multiple biological activities, including anti-cyclooxygenase, anti-oxidant, and anti-inflammatory activity [25–27], hence may have a therapeutic potential in treatment or prevention of several inflammatory diseases, cancer and ageing [28,29].

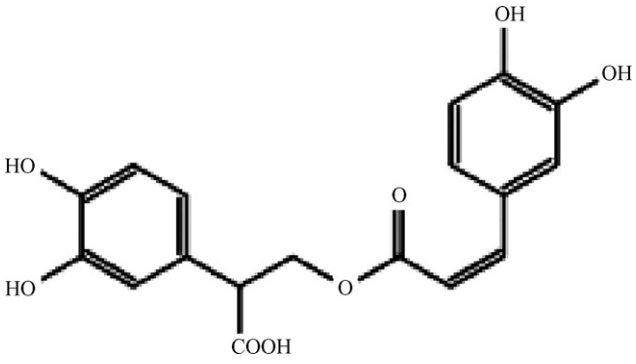

Fig. 1 – Chemical structure of rosmarinic acid.

The rationale for this study stems from previous studies describing that cyclooxygenase-2 (COX-2) induction and PGE_2 level is markedly reduced by pretreatment with RA [26,30] and several anti-oxidants inhibit IDO in IFN-γ-activated macrophages through the posttranslational or transcriptional regulation of IDO expression [31]. In the current study, we examined the modulatory effect of RA on the IDO expression and activity both in IFN-γ-stimulated murine BMDCs and in CD8α^+ DC of tumor-draining lymph nodes and spleen by using the murine tumor model.

2. Materials and methods

2.1. Animals

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 (TCR) transgenic mice in the C57BL/6 background were purchased form The Jackson Laboratory (Bar Harbor, ME, USA). They were bred using hemizygos males and wild-type females. To screen for transgenic offspring, 1 μl blood was collected from the tail, immunostained with FITC-labeled anti-Vα2 and Cy5-labeled anti-CD8 monoclonal antibody (Ab), and analyzed by 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.

2.2. Reagents and antibodies (Abs)

Recombinant mouse (rm) GM-CSF, rm IL-4 and rm INF-γ were purchased from R&D Systems (Minneapolis, MN, USA). Rosmarinic acid (RA, 97%), 1-methyl-DL-tryptophan (1-MT) was purchased from Sigma–Aldrich (St. Louis, MO, USA). InSolution^{TM} JAK Inhibitor I was purchased from Calbiochem (La Jolla, CA, USA). To detect the expression of intracellular IDO in CD11c$^+$CD8α$^+$ DC by flow cytometry, we used mouse anti-IDO monoclonal Ab (Chemicon International, Temecula, CA, USA) as primary Ab and FITC-conjugated goat anti-mouse IgG as secondary Ab (Chemicon International, Temecula, CA, USA). PE-, or Cy5-conjugated mouse Abs used to detect the expression of CD11c (HL3) and CD8α subset were purchased from BD Pharmingen (San Diego, CA, USA). To detect protein levels by western blot, anti-phospho-PKR, anti-phospho-STAT1, anti-STAT1, and anti-β-actin was purchased from Cell Signaling (Beverly, MA, USA), and polyclonal goat anti-mouse IDO Ab was purchased from Santa Cruz Biotechnology, Inc. (CA, USA).

2.3. Generation of BM-derived murine DCs

DCs were generated from murine BM cells, as described by Inaba et al. [32,33] with modifications. Briefly, BM was flushed

from the tibiae and femurs of C57BL/6 and depleted of red cells with ammonium chloride. The cells were plated in six-well culture plates (10^6 cells/ml; 3 ml per well) in OptiMEM (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-ME, 10 mM HEPES (pH 7.4), 20 ng/ml rm GM-CSF and rm IL-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 5 or 7 of the culture, nonadherent cells and loosely adherent proliferating DCs aggregates were harvested for analysis or stimulation, or, in some experiments, replated in 60-mm dishes (10^6 cells/ml; 5 ml per dish). On day 7, 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 mAb (Miltenyi Biotec, Germany) followed by positive selection through paramagnetic columns (LS columns; Miltenyi Biotec, Germany) according to the manufacturer's instructions. The purity of the selected cell fraction was >90%.

2.4. Reverse transcriptase PCR (RT-PCR) analysis of IDO mRNA

Total RNA from 5×10^6 cells was rapidly isolated using the Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA (5 μg) was used for the synthesis of the first strand of cDNA. Amplication of cDNA was performed using forward (5′-GTACATCACCATGGCGTATG-3′) and reverse (5′-GCTTTCGTCAAGTCTTCATTG-3′) oligonucleotide primers. Cycling conditions were 94 °C for 5 min (1 cycle); 94 °C for 30 s, 53 °C for 40 s, 72 °C for 40 s (35 cycles); and 72 °C for 10 min (1 cycle). PCR products of the expected size (740 bp) were fractionated on a 1.5% agarose-TBE gel containing ethidium bromide and were visualized by ultraviolet fluorescence. For references, we quantified mouse GAPDH gene.

2.5. Western blot analysis

The each cell lysates (30 μg of protein/lane) were subjected to electrophoresis on 10% (w/v) polyacrylamide gel in the presence of 0.1% SDS, and then transferred on to nitrocellulose membrane. After blocking of the nitrocellulose membrane with PBS containing 5% skimmed milk for 1 h at room temperature, it was soaked in PBS containing anti-mouse IDO polyclonal antibody. The immunized rabbit serum was diluted to 2000-fold with PBS plus 0.05% (w/v) Tween 20 (PBST). The membrane was then washed extensively with PBST and incubated with horseradish peroxidase-conjugated anti-goat IgG for 1 h at room temperature. Finally, the membrane was washed extensively with PBST and developed with ECL Western blotting detection reagent (Amersham, Little Chalfont, Bucks, UK).

2.6. Enzymatic assay for IDO activity

IDO activity was assayed by the colorimetric method with minor modifications. Briefly, 2×10^6 cells were disrupted by freezing and thawing, the lysate (250 μl) was cleared by centrifugation, and an equal amount of 2× IDO buffer (100 mM PBS, pH 6.5, with 40 mM ascorbate, 20 μM methylene blue, 200 μg/ml catalase, and 800 mM L-tryptophan, all reagents from Sigma–Aldrich, St. Louis, MO, USA) was added. After 30 min at 37 °C, 100 μl of 30% trichloroacetic acid was added to stop the reaction, and a further incubation for 30 min at 52 °C was performed. 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 absorbance was read at 490 nm in a spectrophotometer. Serial dilutions of L-kynurenine were used as standards. One unit of IDO activity was defined as the amount of enzyme producing 1 nmol/h kynurenine. The amount of protein in the samples was assayed by the Bradford method, using the Bio-Rad Protein Assay with BSA as standard.

2.7. Mixed lymphocyte reaction (MLR)

Transgenic OVA-specific $CD8^+$ T cells were purified from bulk splenocytes via negative selection using a mouse $CD8^+$ T cell kit (Miltenyi Biotec, Germany). They were mainly composed of $CD8^+$ cells (>93%) when determined by staining with Cy5-conjugated anti-CD8 antibody (BD Pharmingen, San Diego, CA, USA) by flow cytometry. The lymphocyte was 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 1 μM CFSE in PBS. After 8 min of shaking at 37 °C, Cells were washed in pure FBS and twice in PBS with 10% FBS. DCs (1×10^4 cells per well) generated from BM-derived monocytes of C57BL/6 were pretreated with 1-MT (600 μM) or RA (100 μM), cells were then incubated for 18 h in the presence or absence of IFN-γ (100 U/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, Denmark) together with DCs (1×10^4 per well). After 72 h 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.

2.8. Chromatin immunoprecipitation (ChIP) assay

Chip assays were carried out using Chromatin immunoprecipitation (ChIP) Assay Kit (Upstate Biotechnology Inc., CA, USA) according to a modification of the manufacturer's instructions. Briefly, 2×10^6 cells were fixed with 1% formaldehyde for 10 min at 37 °C to crosslink the protein–DNA complex. Then, they were harvested and washed twice using ice cold PBS containing protease inhibitors (1 mM PMSF, 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 were contained PMSF and protease inhibitor cocktail. Cells were added with SDS lysis buffer and incubated on ice for 10 min. Cell lysates were sonicated to shear DNA to length between 200 and 1000 bp and centrifugated at 14,000 rpm for 10 min at 4 °C. The sonicated cell supernatant 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, 167 mM NaCl) and were precleared with a 27% suspension of protein A agarose/salmon sperm DNA for 30 min at 4 °C with agitation. After Preclearing, the supernatant was recovered after pelleting agarose by centrifugation and incubated with 5 μg of a rabbit specific antibody against STAT1 (Santa Cruz Biotechnology, Inc., CA, USA) overnight at 4 °C. The antibody–protein–DNA complexes were collected adding protein A agarose/salmon sperm DNA for 1 h at 4 °C with agitation. Immunoprecipitated antibody–protein–DNA complexes were washed three times with wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl), and 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 crosslinking, 5 M NaCl was added to each combined elute to a final concentration of 0.3 M, followed by heating at 65 °C for 5 h. Proteins were digested with 100 μg/ml proteinase K for 1 h at 45 °C, DNA was extracted by using QIAquick PCR purification kit (Qiagen, CA, USA). Precipitated DNA fragments were amplicated by PCR. The sequences of *IRF-1* promoter primer are as follows: IRF-1 forward primer 5′-CTTTCCAACACAGGCAAG-3′ and IRF-1 reverse primer 5′-ACTGTGAAAGCACGTAC-3′.

2.9. Analysis of intracellular IDO expression in tumor-bearing mice by flow cytometry in vivo

For analysis of intracellular IDO expression of $CD11c^+CD8\alpha^+$ DCs in spleen and tumor-draining lymph nodes of B16 melanoma using flow cytometry, 6-week-old, male C57BL/6 mice were intraperitoneally injected with RA (5 mg/kg) a day's interval until termination of experiment at 16 days. On day 6, we injected subcutaneously 1×10^6 B16 melanoma tumor cell in the right flank of C57BL/6 mice. On day 16, tumor-draining lymph nodes and spleens from the mice were taken out. Their tumor-draining lymph nodes and spleens were disrupted 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. For intracellular IDO staining, we used a BD Cytofix/Cytoperm Kit (BD Pharmingen, San Diego, CA, USA) following the manufacturer's instructions. Intracellular IDO protein was detected with mouse anti-human IDO monoclonal Abs which react with human and mouse (as primary Abs; CHEMICON International, Inc., USA) and FITC conjugate-goat anti-mouse IgG (as a secondary antibody; CHEMICON International, Inc., USA). For the analysis of surface molecules, cells were stained with Cy5 anti-mouse CD8α (Ly-2), phycoerythrin (PE)-conjugated anti-CD11c. The stained cells were analyzed using a FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA).

2.10. Statistics

Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the mean ± 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. RA inhibits the expression of IDO mRNA and protein induced by IFN-γ in murine BMDCs

In the first series of experiments, we performed to identify whether or not IDO can be expressed within BMDCs stimulated with IFN-γ. On day 5, IDO mRNA was absent in monocytes-derived iDCs, and iDCs stimulated with IFN-γ were also present at low levels of IDO mRNA. However, on day 7 of the culture, iDCs stimulated with IFN-γ highly increased the

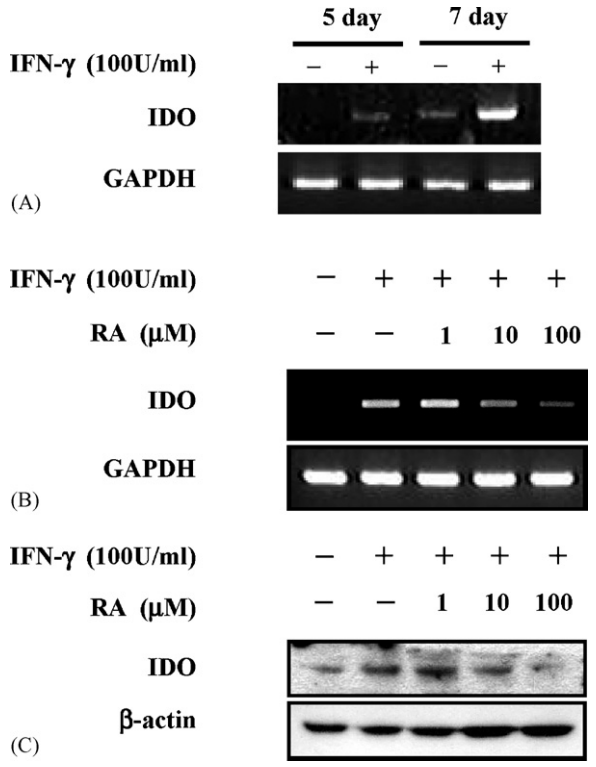

Fig. 2 – Effect of RA on IDO expression in murine BMDCs stimulated by IFN-γ. (A) Murine BM-derived iDCs were generated for 5 or 7 days with GM-CSF and IL-4 and cultured either in medium alone, or exposed to IFN-γ (100 U/ml) for 18 h. (B) iDCs were incubated for 2 h in the presence of 1, 10, and 100 μM RA before the stimulation with IFN-γ and further culture for 18 h. The relative expression levels of IDO were measured using quantitative RT-PCR. (C) IDO protein was detected by western blot in cell extracts using a polyclonal anti-mouse IDO antibody. The cell number from which the protein was derived was normalized prior to loading the gel and Ponceau Red staining of the membrane confirmed that equivalent protein content was being analyzed. β-Actin, housekeeping protein, is shown as a loading control. The data are representative of three-independent experiments.

expression of IDO mRNA (Fig. 2A). We used BMDCs at day 7 of cultures for the rest of the experiment.

We assessed the inhibitory effect of different concentrations of RA on the IFN-γ-induced expression of IDO. We observed that RA significantly reduced the IDO mRNA level in a dose-dependent manner in BMDCs stimulated with IFN-γ (Fig. 2B).

To verify whether RA could affect the expression of IDO protein in BMDCs, we examined IDO protein expression by using a polyclonal mouse IDO Ab. Similar to the results of mRNA, we also found that the expression of IDO protein was reduced by following RA treatment in a dose-dependent pattern (Fig. 2C).

We also confirmed that the inhibitory effect of RA upon IDO expression was not due to cytotoxicity, because there were no remarkable differences in the percentage of dead cells at the concentration of RA $\leq$ 200 μM compared to that of the control (medium only) as assessed by annexin-V and PI staining (data not shown).

3.2. *RA inhibits IDO activity induced by IFN-γ in murine BMDCs*

To determine the inhibitory effect of RA for IDO activity in IFN-γ-activated BMDCs, each cells pretreated with various concentrations of RA were assayed by the detection of kynurenine, the by-product of tryptophan, as described under Section 2. Kynurenine standard curve was obtained

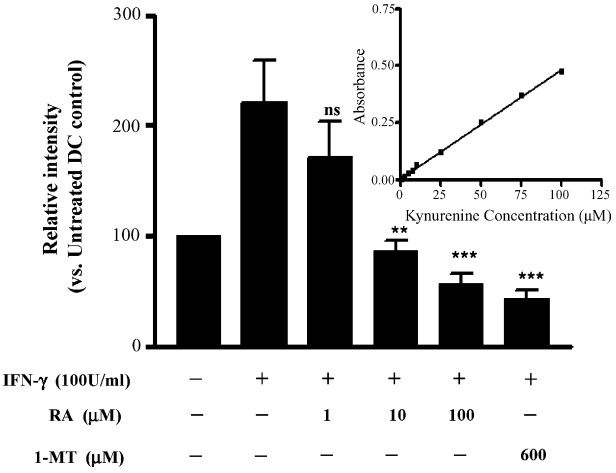

Fig. 3 – Effect of RA on functional activity of IDO in BMDCs stimulated by IFN-γ. The BMDCs were preincubated for 2 h with RA at a range of concentrations to 1–100 μM, and either stimulated for 18–24 h with IFN-γ (100 U/ml) or media alone. Each cell was harvested after incubation and whole-cell lysates were analyzed. For the monitoring IDO functional activity, colorimetric assay was taken. IDO activity was determined via kynurenine formation as described under Section 2, and calculated as a percentage with respect to control (untreated DCs). Values are mean ± S.E.M. of three experiments performed. The asterisk indicates a significant decrease compared to BMDCs stimulated with IFN-γ at $^{**}p < 0.01$, $^{***}p < 0.001$. Inset: Kynurenine standard curve obtained using the absorbance method.

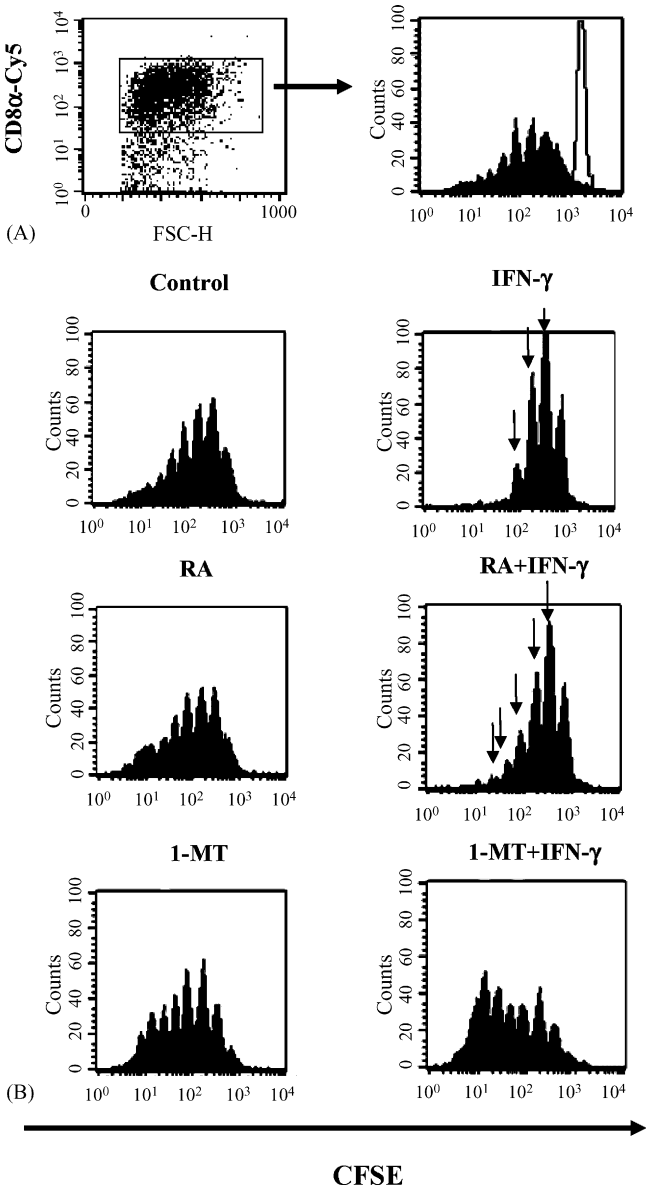

Fig. 4 – Effect of RA on T cell suppression induced by IDO *in vitro*. Transgenic OVA-specific $CD8^+$ T cells were isolated and stained with 1 μM CFSE as described in Section 2. OVA peptide-pulsed DCs were added to 1×10^5 OT-1 T cells for 72 h at a T cell:DC ratio of 10:1 and T cell proliferation was assessed by flow cytometric analysis of CFSE dilution. (A) Flow histograms of CFSE fluorescence for $CD8^+$ gated T cells are shown following T cell stimulation with OVA peptide-pulsed DCs (dark histogram) or nonpulsed DCs (light histogram). (B) DCs were preincubated for 2 h with unsupplemented media, RA or 1-MT as indicated and either stimulated for 18 h with IFN-γ (100 U/ml) or media alone. Each DCs was then treated for 1 h with OVA peptide and co-cultured with T cell for 72 h. Arrows indicate evidence of cell division. Data presented are representative of three experiments yielding similar results.

by measuring the absorbance of kynurenine solutions with final concentrations of 0–100 μM at 480 nm (Fig. 3, inset). Fig. 3 demonstrates that RA dose dependently inhibited IDO activity in murine BMDCs acrivated with IFN-γ similar to 1-MT. Especially, IDO activity in BMDCs treated with 100 μM RA is remarkably reduced compared to that in BMDCs treated with only IFN-γ.

3.3. RA restores the suppression of T cell proliferation induced by IDO in vitro

To evaluate effect of RA on the suppression of T cell proliferation by IDO, we established MLR system using OVA-induced proliferation of CFSE-labeled OT-1 cells as described in Section 2. Proliferation of transgenic OVA-specific $CD8^+$ T cells incubated with DCs stimulated by IFN-γ only was significantly blocked compared to untreated DCs control (Fig. 4B). This suppression was remarkably restored in DC group treated with the tryptophan analogue 1-MT, a well-established competitive inhibitor of IDO. Likewise, the generation of OVA-specific OT-1 cells co-cultured with BMDCs pretreated with 100 μM RA before the stimulation by IFN-γ was moderately increased (Fig. 4B).

3.4. RA inhibits activation and binding of STAT1 to the IRF-1 promoter in response to IFN-γ

Here, to confirm that STAT1 activation is directly involved in IFN-γ-mediated induction of IDO, we determined whether block of STAT1 phosphorylation by specific JAK inhibitor in BMDC could inhibit IDO expression. We observed that JAK inhibitor reduced the IDO mRNA level in BMDCs stimulated with IFN-γ as detected by RT-PCR (Fig. 5A). Subsequently, we sought to determine whether RA would affect STAT1 or PKR phosphorylation when BMDCs are stimulated by IFN-γ. The inhibitory effect of RA on PKR phosphorylation was no remarkable differences compared to PKR phosphorylation in BMDCs treated with IFN-γ only (Fig. 5B). Meanwhile, in response to IFN-γ stimulation, STAT1 phosphorylation reached a plateau at 30 min after IFN-γ stimulation (Fig. 6A). In the case of the dose-dependent manner RA pretreatment, the level of STAT1 phosphorylation in $CD11c^+$ DCs, which harvested at 30 min after IFN-γ stimulation, was dramatically reduced (Fig. 6B).

STAT1 and IRF-1 are involved in the induction of IDO following IFN-γ treatment. STAT1 phosphorylated by IFN-γ-induced reactions binds to the GAS regions in *IDO* promoter as well as *IRF-1* promoter. This issue was prompt us to determine whether binding of STAT1 to *IRF-1* promoter was altered by RA treatment. To address this issue, we performed ChIP assays. BMDCs were incubated in the presence or absence of IFN-γ for 1 h with or without various concentration of RA pretreatment. Chromatin was isolated and immunoprecipitated with STAT1 specific antibody. Immunoprecipitated chromatin was then subject to PCR with primers corresponding to the promoter of *IRF-1*. IFN-γ treatment of BMDCs induced the binding of STAT1 to the *IRF-1* promoter. In remarkable contrast, RA pretreatment (100 μM) of BMDCs completely inhibited the binding of STAT1 to the *IRF-1* promoter (Fig. 6C).

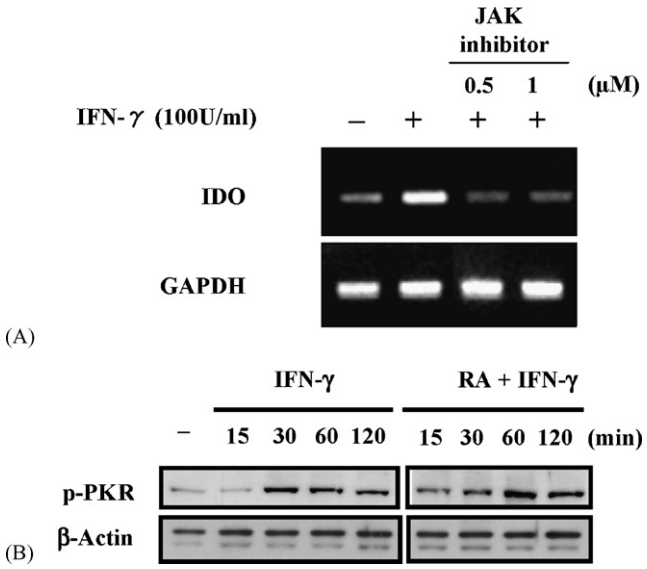

Fig. 5 – Effect of JAK1 inhibitor on IDO specific mRNA expression and effect of RA on phosphorylation of PKR in IFN-γ stimulated DCs. $CD11c^+$ DCs were generated and isolated using MAC $CD11c^+$ beads. (A) Cells were pretreated with or without JAK inhibitor for 1 h as indicated concentration and then with IFN-γ for 18 h. The relative expression levels of IDO were measured using quantitative RT-PCR. (B) Before incubation with 100 U/ml IFN-γ, Purified $CD11c^+$ DCs were pretreated with or without 100 μM RA and harvested at the indicated time periods. Cells were lysed, and the lysate was resolved by SDS-PAGE and probe with anti-phospho-PKR. β-Actin was used as a loading control. The data are representative of three-independent experiments.

3.5. RA inhibits the expression of IDO in $CD11c^+CD8\alpha^+$ DCs of tumor-draining lymph node and spleen of tumor-bearing mice

To further study the inhibitory effect of RA on the expression of IDO *in vivo* tumor-bearing mice, we next assessed the intracellular IDO expression in $CD11c^+CD8\alpha^+$ DCs of tumor-draining lymph nodes and spleen. C57BL/6 mice were injected intraperitoneally with RA (5 mg/kg) a day's interval until termination of experiment. On day 6, B16 mouse melanoma cells were injected subcutaneously in the right flank of C57BL/6 mice, and then IDO expression was analyzed by flow cytometry using anti-IDO, CD11c, and CD8α Abs staining. As might have been expected, the IDO expression increased in spleen (19% of IDO expressing $CD11c^+CD8\alpha^+$ DCs) and tumor-draining lymph node (6.4% of IDO expressing $CD11c^+CD8\alpha^+$ DCs) of B16 melanoma tumor-bearing mice compared with normal control (spleen and lymph node, 12.4% and 4.1%). Although the inhibitory effect of RA on IDO was observed a little in $CD8\alpha^+$ DCs of tumor-draining lymph node (Fig. 7B; 4.5% of IDO expressing cell), a significant reduction of the IDO expression was observed in splenic $CD8\alpha^+$ DCs of mice administrated with RA (Fig. 7A; 11.4% of IDO expressing cell). Similar to the inhibition effect of RA to the expression of IDO in

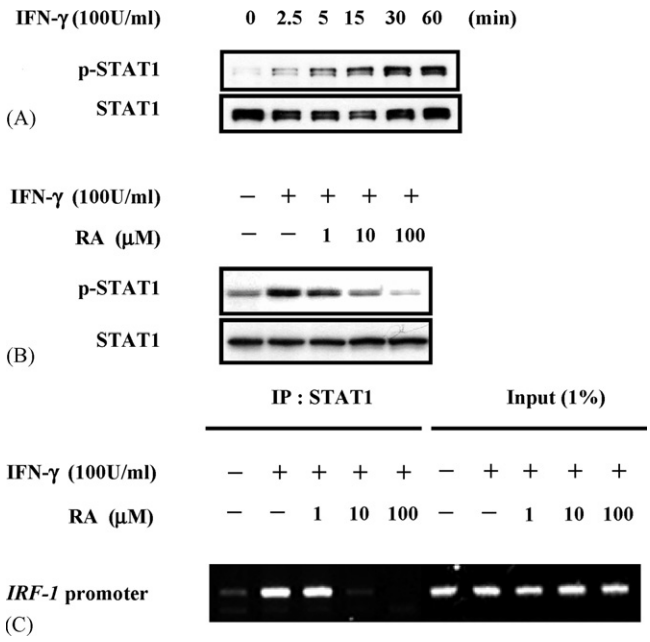

Fig. 6 – RA inhibits the phosphorylation and binding of STAT1 to the *IRF-1* promoter in IFN-γ stimulated DCs. $CD11c^+$ DCs were generated and isolated using MAC $CD11c^+$ beads. Cells were starved in OptiMEM without FBS for 3 h before stimulation with IFN-γ (100 U/ml). (A) Cells were harvested at the indicated time periods, and were subject to western blot analysis. To ascertain that the total level of each STAT1 did not change, the blots were stripped and reprobed with anti-STAT1 Abs raised against the corresponding phosphorylation-independent STAT1. (B) Purified $CD11c^+$ DCs were treated with the indicated concentrations of RA before they were incubated with 100 U/ml IFN-γ. Each cell was harvested after incubation with IFN-γ for 30 min. Whole-cell lysates were analyzed by immunoblot analysis using anti-phospho-STAT1 mouse Abs. (C) ChIP assay was performed after immunoprecipitation of chromatin with STAT1 antibody. Immunoprecipitated chromatin and 1% of input chromatin used for each imunoprecipitate were subjected to PCR with primers specific for the *IRF-1* promoter. This figure is representative of multiple experiments.

BMDC *in vitro*, RA inhibited IDO expression in $CD11c^+CD8\alpha^+$ DCs of the spleen and tumor-draining lymph compared to that of B16 melanoma tumor-bearing mice (Fig. 7).

4. Discussion

IDO is a unique enzyme in that it utilizes a superoxide anion radical as both a substrate and a co-factor. Some anti-oxidants showed inhibitory effects of IDO activity or expression [31]. We already reported that various phytochemicals have strong immunoregulatory activities *in vitro* and *in vivo*, especially on DC [35–40]. We here chose to focus on whether RA, which has anti-oxidant activity as a potent scavenger to the superoxide anion radical [41] and anti-cyclooxygenase activity, could or could not inhibit the functional expression of IDO *in vitro* and *in vivo*. In line with previous studies related to anti-oxidants and IDO modulation, we demonstrated that RA considerably inhibits the IDO expression and its functional activity in IFN-γ stimulated BMDCs in a dose-dependent manner.

Enhanced expression of IDO on tumor-associated APCs inhibits T cell response to tumor antigens by suppressing T cell priming in tumor-draining lymph node [42]. In this study, because RA inhibits directly IDO expression and activity in IFN-γ treated monocyte-derived DCs, it is conceivable that IDO-dependent T cell suppression is blocked by RA treatment *in vitro* analyzed by MLR system using co-cultures of transgenic OVA-specific $CD8^+$ T cells with DCs. Fig. 3 demonstrates that RA restored the IDO-dependent T cell suppression provoked by IDO induction in IFN-γ stimulated BMDCs. However, Won et al. [43] has shown that RA inhibited TCR-induced T cell activation and proliferation in an Lck-dependent manner. This result seems to be a contradictory to our own. This discrepancy may derive from the difference of experimental model system. In the present study, we cultivated T cells with RA-pretreated DCs for MLR, meanwhile Won et al. cultivated T cells in media containing RA without DCs.

As above described results, we prompted to focus on the regulatory mechanisms how RA efficiently inhibits IDO expression in BMDCs stimulated with IFN-γ. Two critical sequence elements, interferon-stimulated response element (IRSE) and interferon-γ activation sequences (GAS) are involved in the response of the *IDO* gene promoter to IFN-γ [23]. Gupta and co-worker [22] showed that the IFN-γ-regulated protein factors, IRF-1 and STAT1, bind to these two sequence elements and interact with each other for an optimal response. STAT1 and IRF-1 as a component of protein complexes formed on *IDO* promoter are required for IDO induction following IFN-γ treatment. When tyrosine phosphorylation of STAT1 was blocked by specific JAK inhibitor, IDO expression in IFN-γ treated BMDCs significantly reduced. Thus, we confirmed that STAT1 phosphorylation is required for the expression of IDO gene in IFN-γ treated BMDCs. Tanscription factor IRF-1 appears to play a critical role in the IFN-γ signal transduction system. IRF-1 mRNA expression is induced at an early stage in response to IFN-γ, peaking after 1 h [44]. This early event is important to IFN-γ action, because changes of IRF-1 expression affect the expression of IFN-inducible genes [45]. PKR and STAT1 have been implicated in the induction of IRF-1 synthesis. Namely, Tyrosine phosphorylation of STAT1 by IFN-γ-induced reactions, perhaps including JAK1/JAK2 and PKR activity, binds to the GAS regions in *IDO* promoter as well as *IRF-1* promoter [46,47]. The results presented here showed that STAT1 phosphorylation was inhibited by RA treatment and this inactivation of STAT1 led to the impaired induction of IRF-1 through inhibition of the binding of STAT1 to the *IRF-1* promoter. Therefore, these results may account for a poor induction of the IDO gene in IFN-γ activated BMDCs by RA pretreatment.

On the other hand, high PGE_2, COX-2 product, in the tumor environment may induce IDO expression. Conversely, inhibiting COX-2/PGE_2 by COX-2 inhibitor specifically reduces IDO expression *in vivo* and *in vitro* [20]. It also has shown that induction of COX-2 and PGE_2 level are decreased by pretreat-

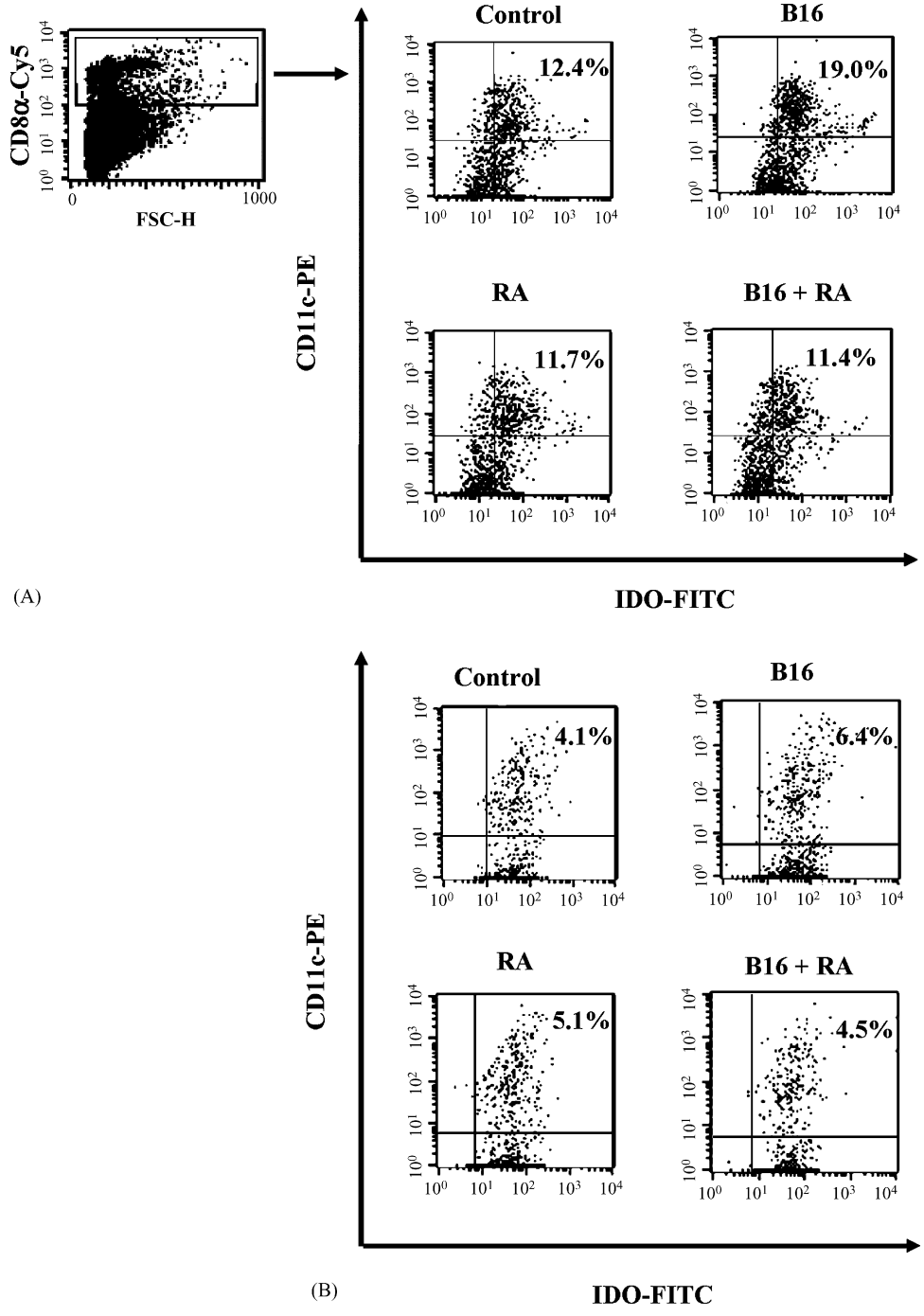

Fig. 7 – RA inhibits the expression of IDO in CD11c$^+$CD8α$^+$ DCs of tumor-draining lymph node and spleen of tumor-bearing mice. C57BL/6 mice were injected intraperitoneally injected with RA (5 mg/kg) a day's interval until termination of experiment at 16 days. On day 6, we injected subcutaneously 1×10^6 B16 melanoma tumor cell in the right flank of C57BL/6 mice. On day 16, spleens (A) and tumor-draining lymph nodes (B) from the mice were taken out. Cells were stained as described in Section 2 and analyzed using a FACSCalibur flow cytometry. Dot plots show the intracellular IDO expression in CD11c$^+$ DCs, the indicated marker for DC, on cells gated for CD8α expression. Numbers indicate the percentage of CD11c$^+$CD8α$^+$ DCs expressing IDO.

ment with RA *in vivo* [48]. We here observed that IDO expression in CD8α$^+$ DCs in spleen and tumor-draining lymph nodes of tumor-bearing mice was reduced by pretreatment with RA. In the context of above described reports, our results may derive from the suppressive activity of RA on COX-2/PGE_2 *in vivo*.

In this paper, we have defined RA as a potent regulator of DCs function, especially on IDO expression in IFN-γ-stimulated murine DCs *in vitro* as well as tumor-bearing mice model. As a result of this effect, we may assume that RA is able to recover IDO-dependent T cell suppression via inhibition of IDO

expression. More specifically, this study may account that RA could inhibit IDO expression by down-regulation of STAT1 activation in IFN-γ-stimulated murine DCs. Taken together, We demonstrated that RA inhibits tolerogenic function of IDO in DCs. And this study suggests that RA is worthy of a new pharmacological agent for IDO regulation.

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