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Resveratrol suppresses tumor progression via the regulation of indoleamine 2,3-dioxygenase

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ABSTRACT

This study showed the potential of resveratrol to inhibit the expression and activity of interferon-γ (IFN-γ)-induced indoleamine 2,3-dioxygenase (IDO) in bone marrow-derived dendritic cells (BMDCs). The mechanism of suppression was associated with the activity of Janus kinase/signal transducers and activators of transcription (JAK/STAT) and protein kinase Cδ (PKCδ). In addition, resveratrol-mediated IDO suppression in IFN-γ-stimulated BMDCs appears to play a pivotal role in anti-tumor activity through the regulation of $CD8^+$ T cell polarization and cytotoxic T lymphocyte (CTL) activity. Systemic administration of resveratrol suppressed tumor growth in EG7 thymoma-bearing mice in an IDO-dependent manner. Taken together, resveratrol not only regulates immune response through the regulation of IDO in a JAK/STAT1- and PKCδ-dependent manner, but also modulates the IDO-mediated immune tolerance in EG7 thymoma.

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1. Introduction

Dendritic cells (DCs) are antigen-presenting cells (APCs) that capture, process, and present antigens to T cells [1]. DCs play pivotal roles in the induction of immunity as well as in the initiation of T cell tolerance [2]. One of the mechanisms responsible for the induction of tolerance involves the expression of the immunoregulatory enzyme indoleamine 2,3-dioxygenase (IDO), which is an essential enzyme that degrades the amino acid tryptophan through the kynurenine pathway [2,3]. IDO functions as a crucial mediator of tumor-mediated immune tolerance by causing T cell suppression, and it is functionally expressed in various immune cells such as DCs and macrophages [2,4,5]. In particular, in many tumors and tolerogenic APCs, IDO degrades tryptophan to kynurenine; this degradation leads to the depletion of tryptophan and consequently, the suppression of T cell proliferation [3]. Recent studies have shown that tumor cells express increased levels of IDO to evade the host immune system [6–8]. Although DCs are crucial for initiating a primary T cell response [9], IDO-positive DCs are thought to be important in the generation and maintenance of a peripheral tolerance through the induction of regulatory T cell responses [10].

Interferon-γ (IFN-γ) is a major inducer of IDO in many cells, particularly in the APCs [11]. Transcriptional induction of the *IDO* gene is mediated by Janus kinase-1 (JAK-1) and signal transducers and activators of transcription (STAT1) [12]. STAT1 acts directly by binding to the IFN-γ-activated sites within the *IDO* promoter, as well as indirectly by inducing IFN regulatory factor-1 (IRF-1), which binds to the *IDO* promoter at 2 IFN-stimulated response element sites (ISREs) [12]. In our previous study, we showed that IFN-γ-induced IDO expression is regulated by the JAK/STAT1 and the protein kinase C (PKC) pathways [13].

Resveratrol (3,5,4′-trihydroxystilbene) is a natural polyphenol that plays a role in a wide range of biological and pharmacological activities, such as anti-cancer, anti-inflammatory, and antioxidant effects in various cell types [14,15]. We previously reported that resveratrol downregulates lipopolysaccharide (LPS)-induced expression of cytokine IL-12 in bone marrow-derived dendritic cells (BMDCs) [16]. Although predominant effects of resveratrol in the physiological environment are well defined, its molecular mechanisms have not been elucidated.

In this study, we showed that resveratrol regulates IFN-γ-induced IDO expression in a JAK/STAT1- and PKCδ-dependent manner. Furthermore, we found that resveratrol modulates IDO-dependent immune escape in EG7 thymoma tumors. On the basis of these findings, we concluded that resveratrol markedly attenuated tumor growth via regulating IDO.

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2. Materials and methods

2.1. Mice

Eight- to ten-week-old male C57BL/6 (H-2K^b and I-A^b) mice were purchased from the Korean Institute of Chemistry Technology (Daejeon, Korea). C57BL/6 OT-1 T cell receptor (TCR) transgenic mice and *IDO*$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, MI, USA). The animals were housed in a specific pathogen-free environment within our animal facility and handled in accordance with the institutional guidelines for animal care.

2.2. Cells and cell culture

The EG7 cell line, an ovalbumin (OVA)-expressing EL4 variant, was purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM L-glutamine (all purchased from Invitrogen, Carlsbad, CA, USA), and maintained at 37 °C in a 5% CO_2 atmosphere.

2.3. Reagents and antibodies

Recombinant mouse (rm) granulocyte macrophage colony-stimulating factor (GM-CSF), rm interleukin-4 (rmIL-4), and rm IFN-γ were purchased from R&D Systems (Minneapolis, MN, USA); resveratrol with a purity of >99% from Sigma–Aldrich (St. Louis, MO, USA); and InSolution™ JAK Inhibitor I and rottlerin from Calbiochem (La Jolla, CA, USA). Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs), which were used for detecting the expression of CD11c (HL3) and CD8 (Lyt-2), were purchased from BD Pharmingen (San Diego, CA, USA). To detect protein levels by Western blotting, anti-phosphotyrosine-STAT1 (Tyr701), anti-phosphoserine-STAT1 (Ser727), and anti-STAT1 were purchased from Cell Signaling (Beverly, MA, USA); polyclonal anti-mouse IDO Ab was purchased from Alexis Biochemicals (San Diego, CA, USA). Polyclonal rabbit anti-mouse Abs against α-tubulin, phospho-PKCδ, and PKCδ were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.4. Generation of murine BMDCs

Primary culture of BMDCs was performed as previously described [17], with slight modification. Briefly, the bone marrow (BM) was flushed from the tibiae and femurs of 6–8-week-old male C57BL/6 mice and was depleted of red blood cells (RBCs) by using Red Blood Cell Lysing Buffer (Sigma–Aldrich, St. Louis, MO, USA). The cells were plated in 6-well culture plates (1×10^6 cells/mL; 2 mL per well) in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 20 ng/mL rmGM-CSF, and 10 ng/mL rmIL-4 and maintained at 37 °C at 5% CO_2 atmosphere. On Days 3 and 5, any floating cells were gently removed from the cultures and replenished with fresh medium. On Day 6 of culture, non-adherent cells and loosely adherent proliferating dendritic cell aggregates were harvested and re-plated in 60-mm dishes (1×10^6 cells/mL; 5 mL/dish) for stimulation and analysis. On Day 7, 80% or more of the non-adherent cells expressed CD11c. In certain experiments, the DCs were labeled with bead-conjugated anti-CD11c mAb (Miltenyi Biotec, Gladbach, Germany) and subjected to positive selection through paramagnetic columns (LS columns; Miltenyi Biotec. Auburn, CA, USA) to obtain highly purified populations for subsequent analysis, according to the manufacturer's instructions. The purity of the selected cell fraction was >90%.

2.5. Western blot analysis

Western blot analysis was performed as previously described [18], with slight modifications. Briefly, cell lysates were subjected to centrifugation at 12,000*g* for 10 min at 4 °C. The resulting supernatants were subjected to SDS–PAGE and transferred to PVDF membranes. The PVDF membranes were then blocked using 5% nonfat milk in a washing buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and incubated with the corresponding antibodies for 1 h at room temperature. The membranes were washed and incubated for 1 h at room temperature with the appropriate secondary antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala). Protein bands were visualized using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.6. Enzymatic assay for IDO Activity

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; Sigma–Aldrich) was added and incubated for 30 min at 37 °C. The reaction was stopped by adding 100 μL of 30% trichloroacetic acid and incubating 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 a wavelength of 480 nm by using a spectrophotometer.

2.7. Mixed lymphocyte reaction

Mixed lymphocyte reaction (MLR) was performed as described elsewhere [19]. Transgenic OVA-specific CD8$^+$ T cells were purified from bulk splenocytes through negative selection using a mouse CD8$^+$ T cell kit (Miltenyi Biotec, Auburn, CA, USA). The obtained cell population was assessed as >93% pure by flow cytometric analysis, after staining with a Cy5-conjugated anti-CD8 Ab. Briefly, the cells were resuspended in 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) in PBS and shaken for 10 min at room temperature. Next, the cells were washed once in pure FBS and twice in PBS with 10% FBS. Immature BMDCs, $OVA_{257-264}$-pulsed BMDCs, $OVA_{257-264}$-pulsed IFN-γ-treated BMDCs, or $OVA_{257-264}$-pulsed IFN-γ + resveratrol-treated BMDCs (1×10^5 cells) were subsequently co-cultured with 1×10^6 CFSE-labeled T lymphocytes in 96-well, U-bottom plates. After 4 days, the cells were harvested, stained with a Cy5-labeled anti-CD8 mAb (to gate OT-1 T cells), and then assessed by flow cytometry.

2.8. In vitro cytotoxicity assays

In mixed cultures, immature BMDCs, $OVA_{257-264}$-pulsed BMDCs, $OVA_{257-264}$-pulsed IFN-γ-treated BMDCs, or $OVA_{257-264}$-pulsed IFN-γ + resveratrol-treated BMDCs (1×10^5 cells) were first cultured with splenocytes of OT-1 TCR transgenic mice (1×10^6 cells per well) for 72 h and then co-cultured with EL4 (1×10^6 cells stained with 1 μM CFSE) or EG7 cells (1×10^6 cells stained with 10 μM CFSE). After 4 h, the mixed lymphocyte tumor cultures were analyzed via flow cytometry.

2.9. Therapeutic implanted tumor experiments

Mice were injected subcutaneously (s.c.) into the right lower back with EG7 thymoma cells (3×10^5 cells), followed by intraperitoneal (i.p.) injection of resveratrol (50 mg/kg) or vehicle

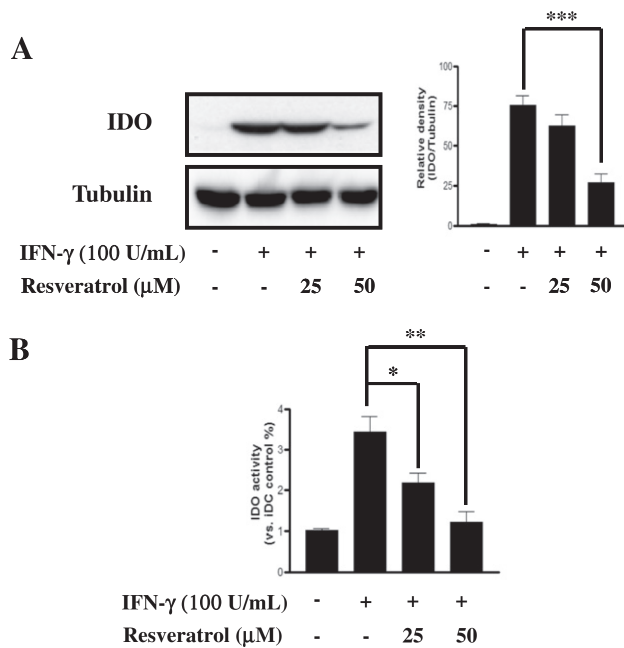

Fig. 1. Resveratrol inhibits IFN-γ-induced expression and activity of IDO in BMDCs. (A and B). BMDCs were pretreated for 1 h in the absence or presence of resveratrol (concentration, 25–50 μM) and incubated with IFN-γ (100 U/mL) for 18 h. The protein levels in the cell extract were detected by, (A) Western blot analysis by using the indicated antibodies. (B) A colorimetric assay was used to monitor IDO activity based on as kynurenine formation, as described under Section 2. Values, presented as percent of control (untreated immature DCs). The mean (SEM) values shown represent 3 independent experiments. *, **, and *** correspond with P values < 0.05, < 0.01, and < 0.001 respectively.

(saline) every 2 days. The tumor size was measured every 4 days, and the tumor mass was calculated using the following equation: Tumor volume (mm^3) = (major axis) × (minor axis)^2 × 0.5236.

2.10. Statistical analysis

All experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the standard error of the mean (SEM). Analysis of variance was used to compare experimental groups with control values, while comparisons between multiple groups were made using Tukey's multiple comparison tests (Prism 3.0 GraphPad software). An unpaired 2-tailed student *t*-test was used to determine statistical significance. A P value < 0.05 was considered to indicate statistical significance.

3. Results and discussion

Previous studies have shown that several antioxidants inhibit the expression of IDO in IFN-γ-stimulated BMDCs [20–22]. This study examined whether resveratrol, a well-known antioxidant, could inhibit the expression of IDO, a representative immune-regulatory protein, in IFN-γ-stimulated murine BMDCs. Resveratrol significantly inhibited IDO expression in a dose-dependent manner (Fig. 1A, upper panel). We also confirmed the resveratrol-mediated inhibition of IFN-γ-induced IDO activity (Fig. 1B, bottom panel). Resveratrol suppressed the expression of IRF-1, which is an essential transcription factor for the IFN-γ-induced IDO expression in BMDCs (Fig. 2A). In a previous study, we showed that the expression of IDO is negatively regulated by curcumin, an anti-oxidant that utilizes the JAK/STAT1 and PKC signaling pathways, in IFN-γ-stimulated BMDCs [13]. Therefore,

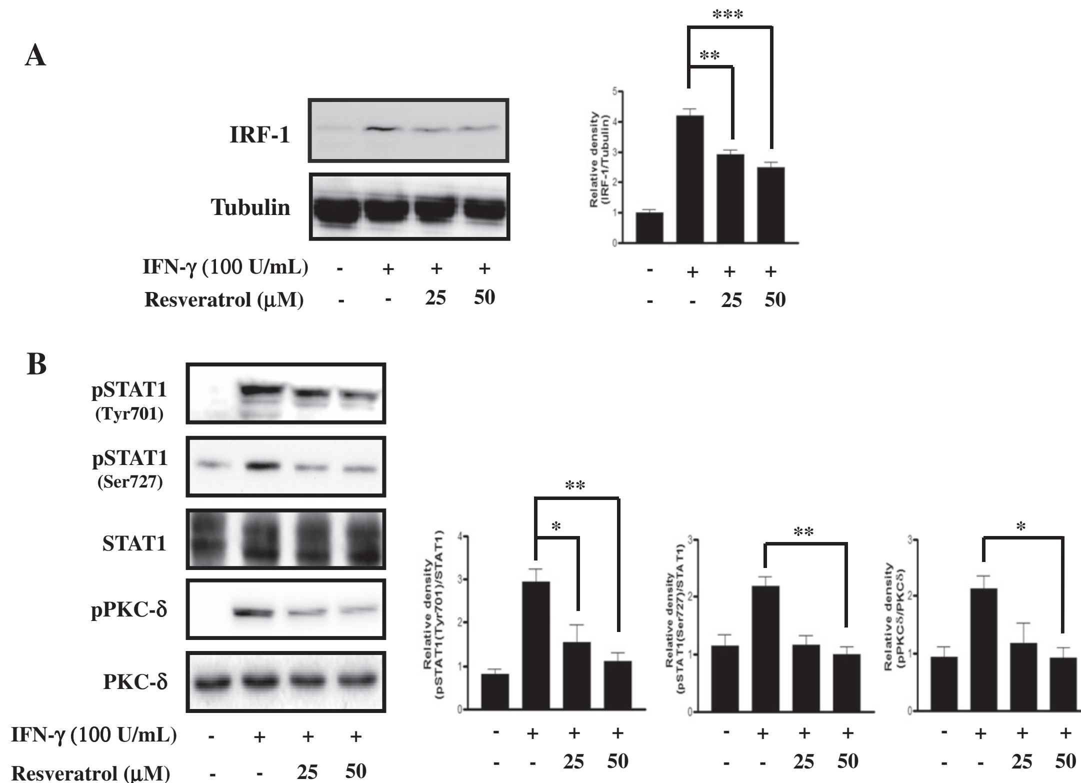

Fig. 2. Resveratrol inhibits IFN-γ-induced expression of IRF-1, JAK/STAT1 activity and PKCδ activity in BMDCs. (A) BMDCs were pretreated for 1 h in the absence or presence of resveratrol (concentration, 25–50 μM) and incubated with IFN-γ (100 U/mL) for 2 h. The mean (SEM) values shown represent 3 independent experiments. ** and *** correspond with P values < 0.01 and < 0.001 respectively. (B) BMDCs were pretreated for 1 h in the absence or presence of resveratrol (concentration, 25–50 μM) and incubated with IFN-γ (100 U/mL) for 30 min. The protein levels in the cell extract were detected by Western blot analysis by using the indicated antibodies. The mean (SEM) values shown represent 3 independent experiments. * and ** correspond with P values < 0.01 and < 0.001 respectively.

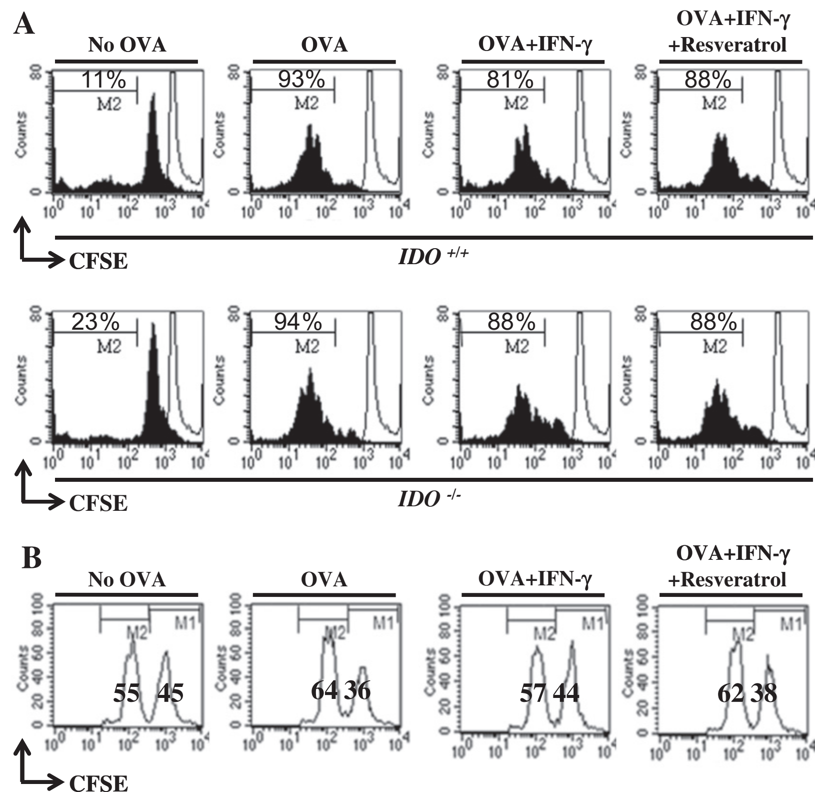

Fig. 3. Resveratrol modulates DC-mediated CD 8+ T cell proliferation and CTL response in an IDO-dependent manner. (A) Immature BMDCs, $OVA_{257-264}$-pulsed BMDCs, $OVA_{257-264}$-pulsed IFN-γ-treated BMDCs, or $OVA_{257-264}$-pulsed IFN-γ + resveratrol-treated BMDCs were cultured with CFSE-labeled splenocytes of OT-1 T cell receptor transgenic mice (1×10^6 cells per well) for 72 h in normal and IDO-absent condition. After 3 days, the cells were harvested and stained with Cy5-labeled anti-CD8 mAb and analyzed by flow cytometry. Histograms showing $CD8^+$ T cell proliferation as assessed by flow cytometry. (B) In mixed cultures, immature BMDCs, $OVA_{257-264}$-pulsed BMDCs, $OVA_{257-264}$-pulsed IFN-γ-treated BMDCs, or $OVA_{257-264}$-pulsed IFN-γ + resveratrol-treated BMDCs were cultured with splenocytes of OT-1 T cell receptor transgenic mice (1×10^6 cells per well) for 72 h and then co-cultured with EL4 (1×10^6 cells stained with 10 μM CFSE) or EG7 (1×10^6 cells stained with 1 μM CFSE) cells. After 4 h, mixed lymphocyte tumor cultures were analyzed by flow cytometry. The percentage in M1 indicates specific lysis of CFSE-stained EG7 cells and that in M2 indicates specific lysis of CFSE-stained EL4 cells.

we investigated the effect of resveratrol on IFN-γ-induced JAK/STAT1 and PKCδ signaling pathways, which are crucial for the expression of IDO in BMDCs. STAT1 and PKCδ were rapidly activated by IFN-γ, but pretreatment of BMDCs with resveratrol showed a significant suppression of the IFN-γ-induced activation of STAT1 and PKCδ (Fig. 2B). These results indicate that resveratrol effectively inhibits the IFN-γ-induced expression of IDO and IRF-1 via the inactivation of the JAK/STAT1- and PKCδ-dependent pathways in BMDCs.

To determine whether the resveratrol-mediated IFN-γ-induced IDO regulation affects T cell proliferation, we performed an MLR assay using $CD8^+$ T cells of OT-1 TCR transgenic mice, which express a TCR specific for the MHC class I-restricted OVA peptide 257–264 antigen ($OVA_{257-264}$) in DCs [23]. The proliferation of CFSE-labeled OVA-specific $CD8^+$ T cells co-cultured with DCs pulsed with $OVA_{257-264}$ was significantly higher than that in T cells co-cultured with non-pulsed DCs (Fig. 3A, upper panel). Treatment with IFN-γ substantially inhibited $CD8^+$ T cell proliferation by $OVA_{257-264}$-pulsed DCs, and resveratrol restored the IFN-γ-mediated suppression of $CD8^+$ T cell proliferation (Fig. 3A, upper panel). To determine whether resveratrol-mediated suppression of $CD8^+$ T cell proliferation in IFN-γ-treated condition is dependent on IDO, we performed an MLR assay by using $IDO^{-/-}$ mice under the same conditions. As expected, IFN-γ-mediated suppression of $CD8^+$ T cell proliferation was not observed in the $IDO^{-/-}$ mice (Fig. 3A, lower panel). In addition, resveratrol did not restore the IFN-γ-mediated suppression of $CD8^+$ T cell proliferation in the absence of IDO (Fig. 3A, lower panel). These results indicate that the resveratrol-mediated IFN-γ-induced modulation of IDO is crucial for $CD8^+$ T cell proliferation by BMDCs pulsed with $OVA_{257-264}$.

Next, we investigated whether resveratrol-mediated modulation of $CD8^+$ T cell proliferation induces the development of cytotoxic T cell responses. The population of CFSE-stained OVA-expressing EG7 tumor cells reduced in the presence of $OVA_{257-264}$-pulsed BMDCs, but not with non-pulsed BMDCs, and IFN-γ inhibits this CTL response (Fig. 3B). Furthermore, the addition of resveratrol restored the CTL activity that was suppressed by IFN-γ (Fig. 3B). However, resveratrol-mediated modulation of DCs did not affect the CTL responses against non-OVA-expressing EL4 tumor cells (Fig. 3B). On the basis of these data, we concluded that resveratrol-mediated modulation of BMDCs potentiates the OVA-specific CTL activity.

After establishing that resveratrol modulated IDO, an immune-regulatory protein, we further investigated whether IDO modulation affected the *in vivo* anti-tumor activity of resveratrol. As shown in Fig. 4A, tumor growth was suppressed in resveratrol-treated mice. In addition, the administration of resveratrol (50 mg/kg) resulted in the suppression of IDO expression in tumor cells isolated from the tumor mass (Fig. 4B). Next, we examined whether resveratrol-induced IDO repression influenced tumor retardation in $IDO^{-/-}$ mice. Our results showed that resveratrol-mediated retardation of tumor growth does not occur in the absence of IDO (Fig. 4A), indicating that resveratrol strongly suppresses tumor growth by reducing the expression of IDO in tumor-bearing mice. On the basis of resveratrol-mediated suppression of IDO in tumor mass, we also inferred that resveratrol blocks

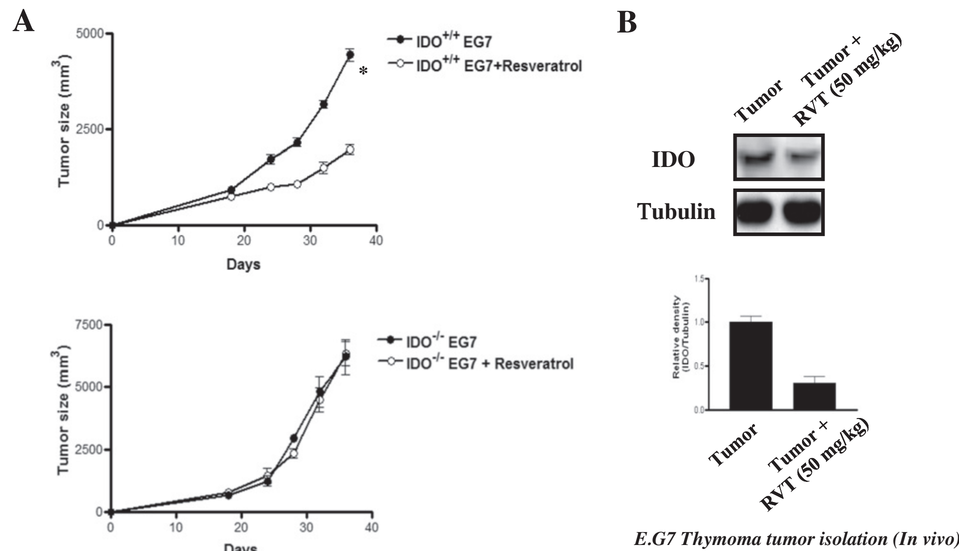

Fig. 4. Resveratrol suppresses tumor growth via IDO regulation. (A) *IDO*$^{+/+}$and *IDO*$^{-/-}$ mice were implanted s.c. with 3×10^5 EG7 thymoma cells in 0.1 mL PBS. They were then injected i.p. with resveratrol (50 mg/kg) every 2 days for 3 weeks. Tumor size was measured using a caliper every 4 days, and tumor volumes were calculated using the formula: Tumor volume (mm^3) = (major axis) $\times$ (minor axis)^2 $\times$ 0.5236. n = 4 mice/group. Significantly different (P < 0.05) from the positive control group as determined by analysis of variance followed by t-test. (B) Isolated tumor cells from tumor mass using CytoSelect™ Clonogenic Tumor Cell Isolation Kit (CELL BIOLABS, INC) were analyzed by Western blot analysis. P value was determined by t-test (P = 0.0025).

the ability of EG7 thymoma tumors to evade the IDO-mediated immune response.

To understand tumor-mediated host immune tolerance, it is important to determine the mechanism of IDO regulation. Tumors can induce tolerance through the modulation of the host immune system, thus allowing inherent evasion of local immune destruction, despite the systemic presence of tumor-reactive T cells [2]. The molecular mechanisms underlying tumor-induced tolerance are still poorly understood. DCs play a pivotal role by functioning as APCs to T cells, which is a key step in the sensitization of naïve T cells to tumor antigens [24]. IDO is expressed in various cells, including DCs and activated macrophages. Its enhanced expression in tumor-associated APCs inhibits the T cell response to tumor antigens by suppressing T cell priming in tumor-draining lymph nodes [25]. By depleting the essential amino acid tryptophan and generating tryptophan catabolites, IDO regulates the immune response via the inhibition of the T cell function [2]. Thus, IDO plays an important role in immune suppression and activation [26,27].

It is well known that resveratrol has crucial roles in a wide range of biological and pharmacological activities. Several *in vitro* and animal model studies reported that resveratrol shows anticancer properties [28–30]. Particularly, resveratrol is known to suppress the development and progression of various cancers through the modulation of multiple pathways for various cellular mechanisms, including apoptosis, cell cycle arrest, and activation of transcription factors [31]. However, the detailed molecular mechanism underlying resveratrol-mediated regulation of IDO remains elusive. This study examined the regulation of IDO by resveratrol in a tumor environment. Generally, it is known that T cells secrete IFN-γ at high levels in tumor environment. In this circumstance, tumor is trying to escape immune surveillance via attacking immune cells. IDO is well known as crucial mediator in this immune disturbance. Thus, we focus IDO, a representative immunosuppressive enzyme, and found that resveratrol regulates immune response through IDO regulation in a JAK/STAT1- and PKCδ-dependent manner. Thus, we inferred that resveratrol-mediated IDO regulation could be a crucial mechanism in the immunogenicity.

Our results have also shown the mechanism of resveratrol-mediated regulation of IDO, in which resveratrol suppressed the expression of IDO in cells isolated from EG7 thymoma tumors in an *in vivo* study (Fig. 4B). IDO-expressing tumor cells enable the tumor to evade the host immune system and thus, its inhibition may serve as a potential mechanism for inducing anti-tumor activity and tumor growth retardation. Recent studies showed that tumor growth suppression could be achieved by inhibiting IDO by using specific inhibitors. Therefore, we speculated that the resveratrol-mediated reduction in the expression of IDO in tumor cells might block the IDO-induced immune escape of the tumor and thus, contribute to tumor growth retardation.

To summarize, the results of this study suggest that resveratrol regulates the DC-mediated immune response via regulating IDO expression in a JAK/STAT1- and PKCδ-dependent manner. In addition, resveratrol not only exerts anti-tumor effect through an IDO-mediated immune modulation in immune cells, but also exerts IDO-mediated tolerance in tumor cells in the tumor environment.

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