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2 # Resveratrol suppresses tumor progression via the regulation of indoleamine
3 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. 22 23 24 25 26 27 28 29 30 31

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35 ## 1. Introduction

36 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]. 37–55

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]. 56–65

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. 66–74

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. 75–80

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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

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