Natural killer cell activation mechanism and anti-cancer effects of resveratrol

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The Graduate School, Yonsei University

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Directed by Professor Jongsun Kim

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ABSTRACT

Natural killer cell activation mechanism and anti-cancer effects of resveratrol

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Natural killer (NK) cells are innate immune lymphocytes that play an important role in anti-viral and anti-tumor immune responses. Additionally, they are suitable targets for cancer immunotherapy owing to their potent cytotoxic activity. Several cancer immunotherapy approaches targeting NK cells are currently in clinical or preclinical development. To activate NK cells, treatments of low dose of IL-2 in combination with other agents are being used in some clinical studies. Unfortunately, these therapies induce toxicity and offset the efficacy of IL-2. To overcome this limitation, studies of therapies involving new cytokines are underway. Therefore, this study aims to find food nutrients that activate NK cells and determine their usefulness as candidates for anti-cancer and anti-metastatic drugs.

In part 1, food nutrients are screened to find NK cell activating molecules. As a result, it was found that resveratrol appeared to activate NK cells most effectively among the substances tested and synergistically increased IFN-γ secretion and NK cells cytotoxicity with interleukin-2 (IL-2). CD107a, NKp30, and NKG2D expression levels were upregulated on the surface of NK cells upon treatment with resveratrol in combination with IL-2 compared with treatment with IL-2 alone. Moreover, NK cell activity in human and mouse whole blood was enhanced upon treatment with resveratrol. Most importantly, administration of resveratrol effectively inhibited tumor growth and metastasis in mice. In conclusion, this research suggests that resveratrol may represent a candidate anti-cancer drug that acts by activating NK cells in vivo.

In part 2, to maximize therapeutic efficacy of cancer immunotherapy, studies are being actively conducted to find therapeutic adjuvants. Resveratrol is one of the well-studied polyphenols which has been shown to possess various potential health benefits, including an anti-tumor effect. The effect of resveratrol as a NK cell booster in vivo tumor model has been previously reported, suggesting that it can be a potential adjuvant for cancer immunotherapies. Also, several studies have demonstrated that resveratrol exerts simultaneous effects on NK cells and T cells. However, the molecular mechanism of how resveratrol activates NK cells is unclear. In this study, the synergistic effect of resveratrol on the enhancement of NK cytolytic activity in combination with IL-2 is confirmed.

Among the proteins mediated by IL-2 signaling, resveratrol activated Akt by regulating the rictor phosphorylation, and mTORC2 via PTEN and S6K1. It was also found that the resveratrol mediated NK cell activation was more dependent on the mTOR pathway than the Akt pathway. Most importantly, resveratrol appeared to increase the expression of c-Myb, which is a downstream transcription factor of Akt and mTORC2. Furthermore, consistent with the previous report, it was shown that c-Myb is essential for NK cell activation induced by resveratrol in combination with IL-2. Taken together, these results demonstrate that resveratrol activates NK cells through Akt and mTORC2-mediated c-Myb upregulation.

Key words: natural killer cell, resveratrol, IFN-γ, NK cytotoxicity, immunotherapy

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I. INTRODUCTION

Natural killer (NK) cells are innate immune lymphocytes accounting for about 15% of all circulating lymphocytes which play a key role in the first line of defense against pathogens and cancer.[1,2] As the name suggests, NK cells spontaneously kill target cells deemed to be dangerous to the host in the absence of prior sensitization; they thus are presumed to be key effectors in cancer immunosurveillance, transplantation rejection, and early viral immunity.[3-5] In addition, NK cells are potent killers of malignant cells and are particularly important in limiting tumor metastasis.[6,7] NK cells can inhibit tumor growth by direct cytotoxicity through the release of cytolytic granules, by secretion of

cytokines such as interferon-gamma (IFN-γ), or indirectly by the orchestration of anti-tumor immune responses.[8] Naïve NK cells can be activated by a variety of pro-inflammatory cytokines, such as IL-2, IL-12, and IL-15.[9-11] These cytokines stimulate NK cell proliferation and enhance NK cell cytotoxicity. When NK cells are activated, they secrete large amounts of IFN-γ, which is essential in viral and tumor clearance and activates the signal transducers and activators of transcription (STAT) signaling pathway in target cells, leading to the improved control of infection.[12,13] IFN-γ production by NK cells restricts tumor angiogenesis, stimulates adaptive immunity, and helps shape T cell responses in lymph nodes.[14] Some clinical studies try to use these cytokines to activate NK cells, such as in IL-2 therapy. However, a high-dose IL-2 therapy can lead to severe adverse effects, including vascular leakage and organ injury caused by activation of the vascular endothelium.[15]

Third-generation immunotherapy represents a new paradigm for cancer treatment.[16] As the focus of treatment has shifted from the tumor itself to the host's immune system, cancer immunotherapy has focused on boosting the body's natural defenses system to fight cancer mainly targeting immune components such as antibodies, T cells, dendritic cells, NK cells, etc.[17-20] Most immunotherapeutic strategies have been focused on T cell responses, but recent studies have tried to use NK cells as potential therapeutic targets in cancer therapy.[21-23] Moreover, it is becoming clear that some of the clinical approaches originally developed to increase T cell cytotoxicity may also activate NK cells, so NK cells have emerged as an effective alternative option to T cell-based immunotherapies, particularly against liquid tumors.[22]

Despite dramatic breakthroughs, clinical studies have exposed obstacles including

low treatment efficacy, low response rate, resistance to immunotherapies, etc.[24-26] To overcome these limitations, researchers are actively working to identify combination treatments, new drug candidates, biomarkers, and therapeutic adjuvants that can boost the therapeutic efficacy of cancer immunotherapy.[27-30] In some clinical studies, the use of a low dose of IL-2, alone or in combination with other agents (such as low-dose IL-2 and IFN-α), has resulted in similar clinical response rates and survival probabilities as the use of high doses of IL-2 alone.[31] Unfortunately, cytokines and other small molecules secreted by IL-2-activated effector cells induce toxicity, which offsets the efficacy of IL-2. To overcome this limitation, studies of therapies involving new cytokines are underway, including the use of IL-2 alone or in combination with IL-2.[32,33]

In the last decade, the field of nutritional immunology has grown steadily. In regard to therapeutic adjuvants for cancer prevention and treatment, foods nutrients, such as resveratrol, curcumin, and pomegranate, are intriguing sources because they have relatively good safety profiles and have shown promising efficacy for combating tumor cells.[34-36] It is hypothesized that, in addition to cytokines, there may be natural substances with the ability to activate NK cells.

In part 1, several compounds are screened, including antioxidants, vitamins, and food ingredients, to identify compounds that can activate NK cells. Furthermore, the potential of one of these compounds, resveratrol, was demonstrated as an anti-cancer and anti-metastatic drug candidate, which may act via NK cell activation. Overall, this research identifies the novel function of resveratrol as a NK booster, which could be a potential adjuvant for cancer immunotherapies. Resveratrol is one of the well-studied

polyphenols which has been shown to possess various beneficial effects, including anti-tumor, anti-inflammation, anti-viral, and anti-fungal and anti-bacterial activity.[37-40] Several examples of the anti-tumor effects of resveratrol have been reported.[37,41] Interestingly, several studies have reported a synergistic effect using resveratrol in combination with doxorubicin in different breast cancer cell lines or apoptosis in colon cancer cell lines.[42,43] Earlier studies have also demonstrated that resveratrol exerts simultaneous effects on NK cells and T cells.[44,45] In part 1, it was shown that resveratrol contributes to enhance NK cell cytotoxicity in vitro as well as in vivo tumor models, suggesting that resveratrol could be used as a potential adjuvant for cancer immunotherapies. However, the molecular mechanisms of how resveratrol activates NK cells remain unclear.

In part 2, the mechanism of resveratrol on NK cell activation was investigated. It was shown that resveratrol activates NK cells in a synergistic manner with IL-2. Among the proteins mediated by IL-2 signaling, resveratrol appeared to activate Akt by regulating mTOR complex 2 (mTORC2) via PTEN and S6K1. Furthermore, it was found that resveratrol increased expression of c-Myb, which is known to play a key role in the regulation of NK cell activity. Together, these results demonstrate that resveratrol activates NK cells as a consequence of causing downstream changes in mTORC2-mediated c-Myb.

II. MATERIALS AND METHODS

1. Cell lines and cell cultures

The human NK cell line NK92 (ATCC, Manassas, VA, USA, CRL-2407™) was maintained in minimum essential medium α (MEM-α) (Gibco, New York, NY, USA) supplemented with 12.5% heat-inactivated fetal bovine serum (FBS; Gibco), 12.5% heat-inactivated horse serum (Gibco), 0.2 mM myo-inositol (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 0.02 mM folic acid (Sigma-Aldrich), 1% penicillin/streptomycin (WelGENE, Gyeongsansi, Korea), and 5 ng/ml IL-2 (ATGen, Sungnamsi, Korea). Human erythroleukemia cell line K562 (ATCC, Manassas, VA, USA, CCL-243™) was maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. Mouse melanoma cell line B16F10 (ATCC, Manassas, VA, USA, CRL-6475™) was maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin.

2. Antibodies and reagents

For western blot, anti-β-actin antibody was purchased from Sigma-Aldrich. Phospho-STAT5 mAb, STAT5 mAb, phospho-Akt mAb, Akt mAb, phospho-Erk mAb, Erk mAb, phospho-PDK1 mAb, PDK1 mAb, phospho-Rictor mAb, Rictor mAb, phospho-S6K1 mAb, S6K1 mAb and c-Myb mAb were purchased from Cell Signaling Technology (Danvers, MA, USA). PTEN was purchased from BD Pharmingen (San

Diego, CA, USA).

For flow cytometry, PE-conjugated anti-human CD107a (555801) and APC-conjugated anti-human CD56 (555518) were purchased from BD Pharmingen. BV421-conjugated anti-human CD3 (300434), APC-Cy7-conjugated anti-human CD16 (302017), PE-conjugated anti-human NKG2D (320806), PE-conjugated anti-human NKp30 (325208), PE-conjugated anti-human NKp44 (325108), and PE-conjugated anti-human NKp46 (331908), BV650-conjugated anti-mouse TCRβ (109251), PE-conjugated anti-mouse NK1.1 (108708), PerCP-Cy5.5-conjugated anti-mouse IFN-γ (505822), and PE-Cy7-conjugated anti-mouse CD107a (121619) were purchased from BioLegend (San Diego, CA, USA).

Resveratrol, 3-amino-1-propanesulfonic acid (3-APS), glutathione, taurine, glycine, alanine, β-alanine, β-carotene, retinoic acid, retinol, retinal, tazarotene, vitamin B1, vitamin B12, vitamin D3, 2,3-butandiol, quercetin, triacetyl resveratrol, piceatannol, and celastrol (c-Myb inhibitor) were purchased from Sigma-Aldrich. Lycopene and catechin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Resveratrol-3-O-glucuronide was purchased from Cayman Chemical (Ann Arbor, MI, USA). Resveratrol-4′-glucuronide was purchased from Spibio (Montigny Le Bretonneux, France). Resveratrol-trisulfate was purchased from Carbosynth (Berkshire, UK). MK-2206 (Akt inhibitor) and KU-0063794 (mTOR inhibitor) were purchased from Selleckchem (Houston, TX).

3. Cell proliferation assay

Cell proliferation was measured using the CCK-8 assay kit (Dojindo Laboratories, Kumamoto, Japan). NK92 cells (1×10^4 cells/well) were dispensed in 100 μl aliquots in triplicate in a 96-well flat bottom plate and incubated for 4 hrs. After incubation, 10 μl of CCK-8 solution was added to each well. After 4 hrs, absorbance was measured at 450 nm using a microplate reader (Epoch, BioTek, USA).

4. Enzyme-linked immunosorbent assay (ELISA) analysis

An IFN-γ ELISA set was purchased from BD Biosciences (San Diego, CA, USA). NK92 cells (5×10^4 cells/well) were dispensed in 500 μl into a 24-well plate and incubated for 36 hrs. Then, the supernatants were harvested and dispensed in triplicate into a 96-well microplate pre-coated with capture antibodies. After incubation for 2 hrs, each well was washed with washing buffer (0.05 % Tween 20 in PBS, pH 7.4), followed by the addition of an HRP-conjugated detection antibody and incubation for 1 hr. The substrate solution 3,3′,5,5′-tetramethylbenzidine (TMB) was added under protection from light. Absorbance was measured at 450 nm using a microplate reader.

5. CD107a degranulation assay

NK92 cells were treated with or without resveratrol and incubated for 36 hrs. After incubation, NK92 cells (2×10^4 cells/well) were dispensed in 100 μl into a 96-well round-bottom plate and incubated with an equal number of K562 target cells. Then, anti-CD107a antibody was added, and the plate was incubated for 4 hrs. Cells were harvested and stained with anti-CD56 antibody for 30 mins on ice. After 2–3 washes

with PBS containing 1% FBS, fluorescence was measured using a BD LSR II flow cytometer (BD Biosciences). PBMCs were treated with or without resveratrol for 24 hrs in the presence of IL-2. After incubation, PBMCs were incubated with K562 cells (5:1 ratio) for 4 hrs. Cells were harvested and stained with anti- CD3, CD16, and CD56 antibody in the presence of fixable live/dead stain (Invitrogen) for 30 mins on ice. After 2–3 washes with PBS containing 1% FBS, fluorescence was measured using a BD LSR II flow cytometer (BD Biosciences).

6. Flow cytometry

NK92 cells (5×10^4 cells/well) were dispensed in 500 μl into a 24-well plate and incubated with or without resveratrol. After incubation, cells were washed twice with PBS containing 1% FBS and stained with fluorochrome-conjugated antibodies in PBS containing 1% FBS for 30 mins on ice in the dark. The cells were washed twice, and then fluorescence was measured using the BD LSR II.

7. Real-time PCR analysis

Total RNA was extracted from NK92 cells using TRIzol (Life Technologies, Carlsbad, CA, USA), and cDNA was synthesized using the SensiFAST cDNA synthesis kit (Bioline, Taunton, MA, USA) according to the manufacturer's instructions. Synthesized cDNAs were used as templates for subsequent PCR amplification of the IFN-γ, NKp30 isoforms, c-Myb, and GAPDH genes. The following primers were used:

IFN-γ,
5′-GTCCAACGCAAAGCAATACA-3′ and 5′-CTCTTCGACCTCGAAACAGC-3′;

NKp30a,
5′-GGTGGTGGAGAAAGAACATC-3′ and 5′-CTTTCCAGGTCAGACATTTGC-3′;
NKp30b,
5′-GGTGGTGGAGAAAGAACATC-3′ and 5′-GAGAGTAGATTTGGCATATTTGC-3′;
NKp30c,
5′-GGTGGTGGAGAAAGAACATC-3′ and 5′-CATGTGACAGTGGCATTTGC-3′;
Myb,
5′-CATGTTCCATACCCTGTAGCG-3′ and 5′-TTCTCGGTTGACATTAGGAGC-3′;
GAPDH,
5′-CAGCCTCAAGATCATCAGCA-3′ and 5′-GTCTTCTGGGTGGCAGTGAT-3′.

Quantitative real-time RT-PCR was carried out using a KAPA SYBR FAST qPCR kit (KAPA Biosystems, Wilmington, MA, USA), and amplification was performed on an ABI Prism StepOnePlusTM detection system (Applied Biosystems, Foster City, CA, USA) according to the conditions recommended by the manufacturer. The experiments were performed in triplicate, and results were normalized to the expression of GAPDH. The relative expression levels of the target genes were calculated by the 2-ΔΔCt method.

8. Cytotoxicity assay

The Calcein-AM (Invitrogen, Carlsbad, CA, USA) assay was used to measure NK cells cytotoxicity against target cells. Calcein-AM was added to target cells to a final concentration of 2 μM and incubated for 30 mins at 37°C and 5% CO2. Calcein-AM-labeled target cells were washed with PBS twice, and then 1×10^4 cells were dispensed in 100 μl aliquots in quadruplicate into a 96-well round-bottom plate. NK cells were

added at different effector:target (E:T) ratios and co-cultured for 4 hrs. The calcein-AM released from lysed target cells was measured with a spectrophotometer (ex: 485 nm/em: 535 nm). Total specific lysis was calculated by the formula:

$$\text{Specific cytotoxicity (\%)} = \frac{\text{Experimental release-spontaneous release}}{\text{Maximum release-spontaneous release}} \times 100$$

9. Ex vivo NK cell activity assay

Blood was obtained from healthy donors after acquiring institutional review board approval and informed consent (No: 4-2019-0007). Animal experiments were approved by the Yonsei University Health System Institutional Animal Care and Use Committee (YUHS-IACUC, No: 2017-0203).

For the human NK cell activity assay, 1 ml of whole blood was incubated with activator (provided as part of the kit) for 24 hrs at 37°C under 5% CO2 (n=13). For the mouse NK cell activity, 100 μl of whole blood was incubated with 30 μl activator for 24 hrs at 37°C (tumor-naïve or tumor-bearing mouse model, n=8). After incubation, the supernatant was collected, and the released IFN-γ was measured using the murine NK Vue Kit (ATGen). Standards and samples were then incubated in an anti-human (or murine) IFN-γ antibody-coated plate for 2 hrs (1 hr for murine) at 20–25°C. After incubation, each well was washed 4 times before the addition of an HRP-conjugated detection antibody and incubation for 1 hr. Each well was washed 7 times (4 times for murine), and then the TMB substrate solution was added in the dark. After incubation for 30 mins, the stop solution was added, and absorbance was measured at 450 nm.

10. Animal studies

Animal experiments were approved by the Yonsei University Health System Institutional Animal Care and Use Committee (YUHS-IACUC, No: 2017-0203). Specific pathogen-free female C57BL/6 mice, 7 wks of age, were purchased from Orient Bio (Seoul, Korea). Mice were maintained in a temperature-controlled, air-conditioned animal house under a 12/12-h light/dark cycle and received food and water ad libitum. Animals were cared for and treated in accordance with procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington, DC, USA). B16F10 cells were used between passages 4 and 5.

For the anti-tumor assay, B16F10 cells (5×10^5 cells) in 0.2 ml of PBS were injected subcutaneously (s.c.) into the right flanks of mice on day 0. On day 18, the mice were sacrificed, and the tumors were dissected. The activity of NK cells isolated from tumors and draining lymph nodes was evaluated by flow cytometry. Tumor volume was calculated for each mouse by measuring the length (L), width (W), and height/diameter (H) of approximately ellipsoid tumors using a caliper, according to the following formula:

$$Tumor\ volume\ (mm3) = L \times W \times H \times 0.52$$

For the pulmonary tumor metastasis assay, B16F10 cells at a concentration of 1×10^5 cells in 0.1 ml of PBS were injected intravenously (i.v.) into the tail veins of mice on day 0. On day 18, the mice were sacrificed, and the lungs were extracted. The melanoma foci on the lung surface were counted macroscopically. The activity of NK cells isolated from lungs was evaluated by flow cytometry.

To evaluate the activity of resveratrol, 0.5 mg/kg of resveratrol in 0.1 ml of PBS

was i.v. injected a total of 6 times, on days –2, 0, 2, 4, 6, and 8 (n=10 or 7).

11. NK cells depletion model

To deplete NK cells, 25 μg anti-NK1.1 monoclonal antibody (PK136) or control mouse IgG2a (both from Bio-X-Cell) were injected intraperitoneal (i.p.) on days -3, 2, and 8. B16F10 cells (5×10^5 cells) in 0.2 ml of PBS were injected subcutaneously into the right flanks of mice on day 0. On day 18, the mice were sacrificed, and the tumors were dissected and tumor volume was calculated.

12. Western blot analysis

After treatment, NK92 cells were harvested and washed. The cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitor cocktail. Protein concentration was determined by BCA protein assay (Thermo Scientific). Sample buffer was added to each sample. Samples were boiled at 100℃ for 5 mins and cooled on ice and then loaded onto a 10% SDS-PAGE gel. After running, gels were transferred to a methanol-activated PVDF membrane and blocked for nonspecific reactivity. The membrane was probed with specific antibodies diluted in TBS-T containing 3% BSA and incubated for overnight at 4℃. The membrane was washed 3 times for 10 mins and incubated for 1 hr with secondary antibodies. Immunoreactivity was detected using an ECL solution (Thermo Scientific).

13. DNA/protein array for promoter binding activity analysis

Nuclear extracts were prepared with a Nuclear Extraction kit (Panomics, Fremont, CA, USA) following the manufacturer's instructions. Each array assay was carried out following the procedure in the Protein/DNA Array kit (Panomics) user's manual. 5 μg of nuclear extract was mixed with DNA probe (10 ng/μl), and the mixture was incubated at 15°C for 30 mins. The reaction was loaded onto the column and incubated for 30 mins on ice. After incubation, the column was washed with washing buffer 4 times followed by centrifugation at 7,000 x g and 4°C. DNA probes were eluted and the reactions were denatured at 95°C for 3 mins then hybridized to the array membrane at 42°C overnight. The membrane was washed twice in washing buffer I at 42°C for 20 mins and then twice in washing buffer II at 42°C for 20 mins. The membrane was detected using chemiluminescent reagents and exposed to photographic film. The array images were acquired and the spot intensities of all the transcription factors detected were measured using Image J software.

14. Evolutionary Conserved Regions (ECRs) Browser

To identify whether a c-Myb binding site existed within the IFN-γ promoter region, an ECR browser, a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes, was used. Annotation of conserved transcription factor binding sites underlying ECR Browser conservation plots were displayed using the 'Synteny/Alignments' link in the top menu, and performed following the procedure in the instructions.

15. c-Myb knockdown using Lentiviral transduction

MYB shRNA plasmids and the control shRNA plasmid were purchased from Sigma Aldrich. Lentivirus particles were produced using a third-generation packaging system. For this system, pMDLg/pRRE (plasmid #12251; Addgene, Cambridge, MA, USA), pRSV-Rev (plasmid #12253; Addgene) and pMD2.G (plasmid #12259; Addgene) were kindly provided by Dr. Didier Trono. To transfect the plasmids into HEK293T cells, Lipofectamine 3000 was used (Invitrogen). For lentiviral transduction, NK-92 cells were stimulated with IL-2 (5 ng/ml) for 1 hr and then infected by mixing with the supernatants containing lentiviral particles and protamine sulfate (15 μg/ml; Sigma Aldrich). To increase the lentiviral transduction efficiency, the mixtures were centrifuged at 360 × g for 90 mins at 32°C. For the selection of transgene-positive cells, the cells were cultured in complete growth medium containing 2 μg/ml puromycin for up to 4 wks.

16. Statistical analysis

Statistical analysis was performed with GraphPad Prism software (GraphPad software, San Diego, CA, USA). Data were expressed as means ± standard error of the mean (SEM). Student's t-test and one or two-way ANOVA were used to compare distributions between groups. $p < 0.05$ was considered statistically significant.

III. RESULTS

Part 1. In vivo anti-cancer effects of resveratrol mediated by NK cell activation

1. Screening of NK cell-activating molecules

To identify a non-cytokine natural substance capable of activating NK cells, several compounds, including known antioxidants, vitamin-based substances, and food ingredients were tested for their ability to activate NK92 cells (Figure 1). In this study, the vehicle is the solvent control which contains the same amount of ethanol used to dissolve each compound. Since NK cells require IL-2 to proliferate, the experiment was conducted in the presence of IL-2. Most of the compounds appeared to have no effect on NK cells viability when added at a concentration of up to 20 μM, whereas retinoic acid and retinal slightly inhibited cell proliferation (data not shown). IFN-γ secretion was then measured to determine whether NK cells were activated by these substances. Interestingly, while most substances appeared to have no effect on IFN-γ secretion, resveratrol significantly increased IL-2-induced IFN-γ secretion about 2-fold compared to that of IL-2 treatment alone suggesting potential roles of resveratrol to activate NK cells (Figure 1).

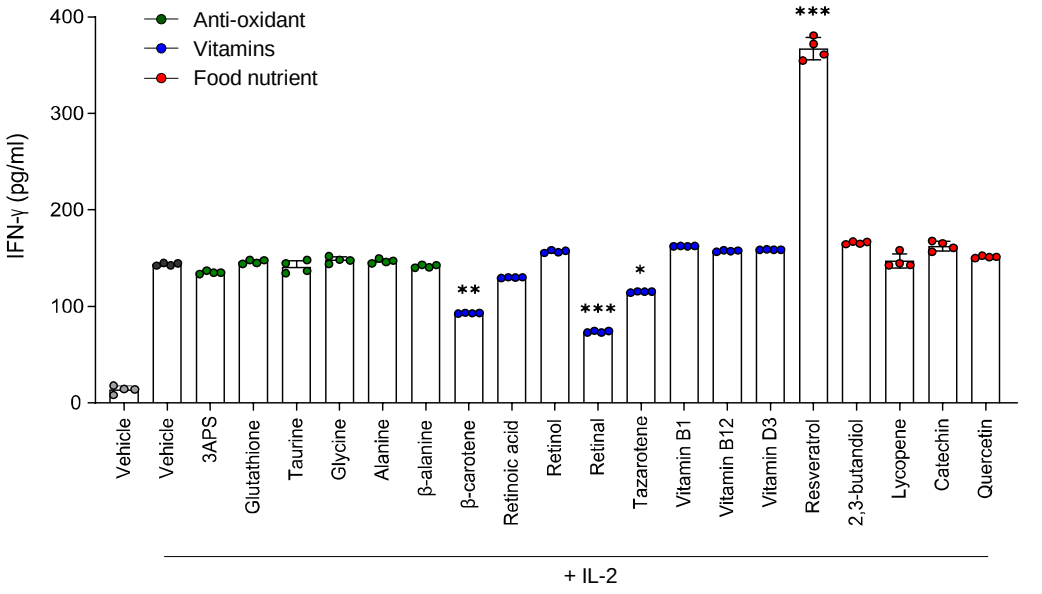


Figure 1. Screening of NK cell-activating molecules. Comparisons of IFN-γ secretion in NK92 cells treated with 20 μM resveratrol and other compounds (with 5 ng/ml of IL-2) for 36 hrs. IFN-γ secretion was measured by ELISA. Data are shown as mean ± SEM of 4 independent experiments. Asterisks indicate statistical significance by one-way ANOVA: *p<0.05, **p<0.01, ***p<0.001, ns: not significant (p>0.05).

2. Resveratrol increased IL-2-induced IFN-γ secretion from NK cells

As mentioned above, among the antioxidants, vitamins, and food nutrients tested, only resveratrol appeared to increase the IFN-γ secretion from NK cells (Figure 1). Moreover, as shown in Figure 2A and 2B, resveratrol did not significantly affect NK cell proliferation but increased IFN-γ secretion in a dose-dependent manner. Particularly, at a resveratrol concentration of 20 μM, IFN-γ secretion was increased by more than 50% compared to that of IL-2 alone-treated cells (Figure 2B). Next, the proliferation of NK cells and secretion of IFN-γ were measured after treatment with resveratrol with or without IL-2 to investigate whether resveratrol is effective as a replacement for IL-2 or has synergistic effects in combination with IL-2. As shown in Figure 3, resveratrol had no effect in the absence of IL-2. Moreover, in the presence of IL-2, the proliferation of NK cells was not affected by resveratrol (Figure 2C). While IFN-γ secretion was not altered for up to 12 hrs after treatment, it thereafter increased by about 40% or more compared to that observed in the group treated with IL-2 alone (Figure 2D). It was also confirmed by real-time PCR that the mRNA level of IFN-γ was upregulated by resveratrol treatment in the presence, but not the absence, of IL-2 (Figure 2E). Altogether, these results indicate that resveratrol has synergistic effects in combination with IL-2 in activating NK cells.

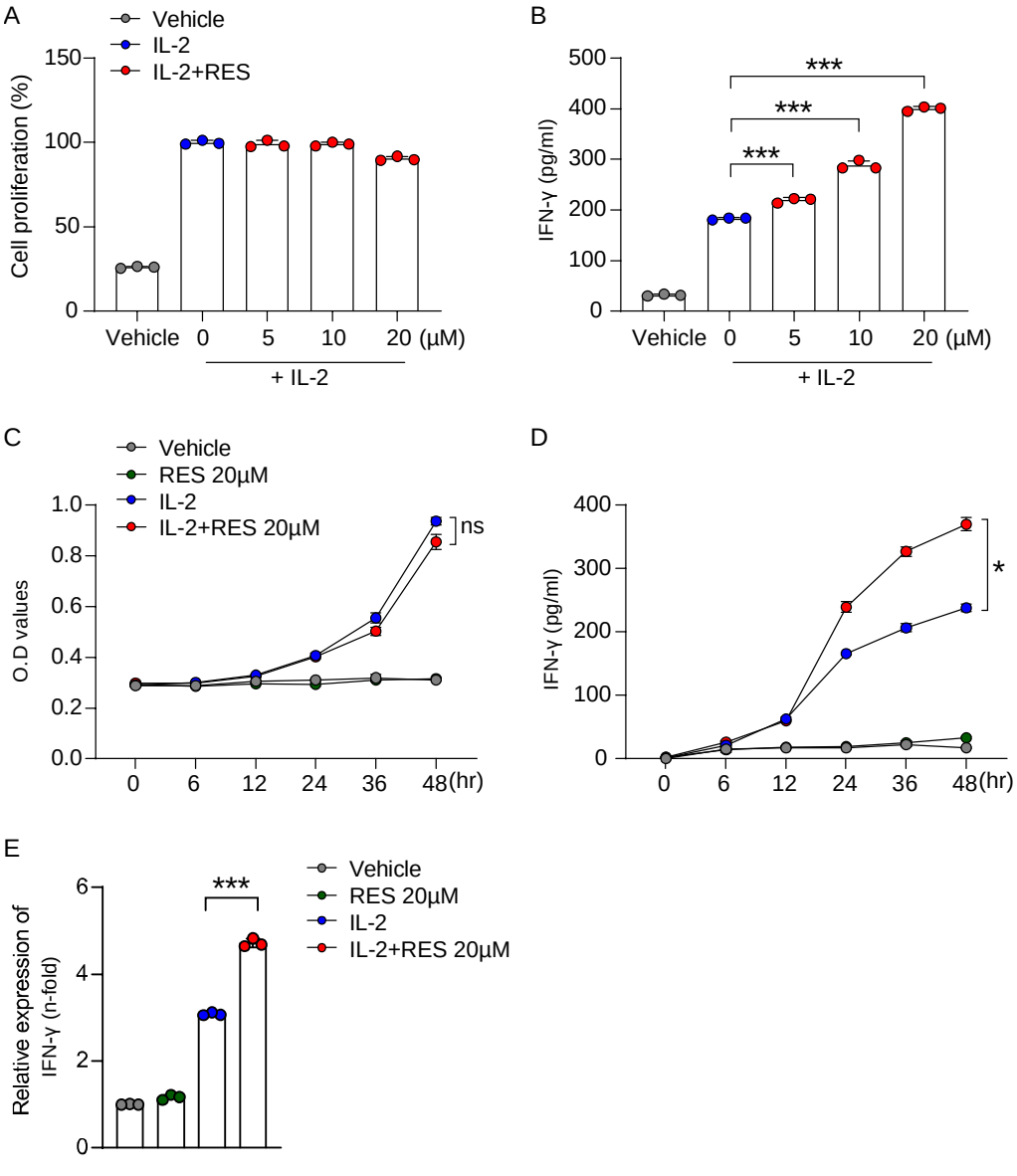
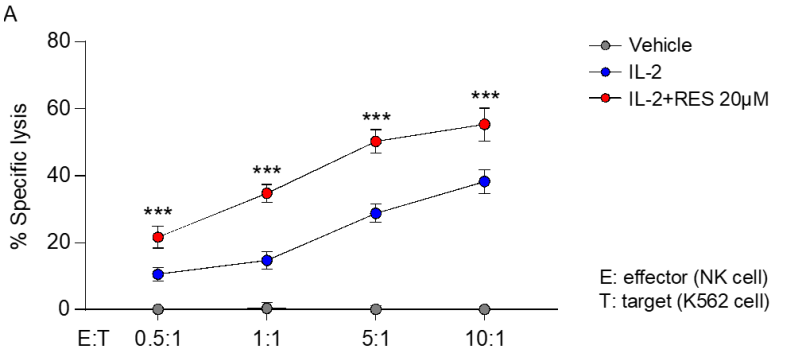


Figure 2. Resveratrol increased IL-2-induced IFN-γ secretion from NK cells. (A-B) NK92 cells were treated with 0–20 μM resveratrol in the presence of 5 ng/ml IL-2 for 36 hrs. (C-D) NK92 cells were treated with 20 μM resveratrol for 0–48 hrs with or without 5 ng/ml IL-2. (E) The mRNA levels of IFN-γ in NK92 cells after treatment of resveratrol for 24 hrs were measured by real-time PCR and calculated by the ΔΔCT method. GAPDH was used as an internal control. Data are shown as mean ± SEM of 3 independent experiments. Asterisks indicate statistical significance by one or two-way ANOVA: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns: not significant ($p>0.05$).

3. Effects of resveratrol on NK cell activity

Next, the effect of resveratrol on NK cells cytolytic activity was examined. Consistent with previous studies, IL-2 treatment significantly enhanced the killing activity of NK cells. Intriguingly, co-treatment of resveratrol together with IL-2 further heightened NK cytotoxicity by 21.3 % compared with IL-2 treated cells at a 1:1 ratio indicating the increase of NK cytotoxicity by resveratrol (Figure 3A). The effect of resveratrol on NK cells degranulation activity was also examined by measuring CD107a expression as a functional marker of NK cell activation.[46] NK cells were co-incubated with K562 target cells, which do not express major histocompatibility complex (MHC) class molecules and are susceptible to NK cell-mediated killing.[47] This was followed by analysis of surface levels of CD107a by flow cytometry. As shown in Figure 3B, after NK cells were pre-treated with IL-2, NK cells expressing CD107a accounted for 18.3±0.23 % of all $CD56^+$ NK cells. CD107a expression on the surface of NK cells pretreated with resveratrol and IL-2 increased 1.8-fold compared to that on the surface of IL-2 alone-pretreated cells, resulting in 32.9±2.59 % of $CD56^+$ NK cells expressing CD107a.



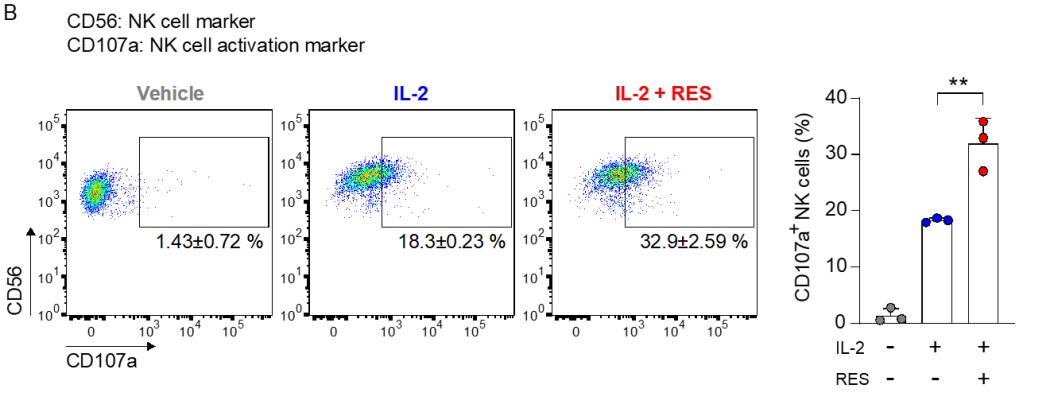


Figure 3. Effects of resveratrol on NK cell activity. (A) NK cells cytotoxicity was determined using calcein-AM. NK92 effector cells were treated with 20 μM resveratrol for 36 hrs, followed by incubation with K562 target cells at different ratios for 4 hrs. (B) CD107a expression on the surface of NK92 cells after resveratrol treatment was measured by flow cytometry. NK92 cells were treated with 20 μM resveratrol for 36 hrs, followed by co-culturing with K562 target cells at a 1:1 ratio for 4 hrs. Quantitative analysis showing the percentages of $CD56^+$ $CD107a^+$ cells. Data are shown as mean ± SEM of 3 independent experiments. Asterisks indicate statistical significance by one-way ANOVA: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns: not significant ($p>0.05$).

4. Effects of resveratrol on NK cell activating receptor expression

Next, the effect of resveratrol on the expression of NK cell-activating receptors, which have central roles in the cytotoxicity of NK cells, was measured. As shown in Figure 4A, the expression of NK cell activating receptors was significantly increased by resveratrol compared to IL-2 alone. Since a previous study has shown the immunomodulatory functions of NKp30 depend on its isoforms,[48] the change of expression level of NKp30 isoforms upon IL-2 with or without resveratrol treatment was analyzed. While resveratrol did not affect the expression of NKp30c, known as an inhibitory Nkp30 isoform, the activating isoforms, including NKp30a as well as NKp30b, were significantly increased by resveratrol (Figure 4B). Altogether, these results suggest that resveratrol might enhance the overall activity of NK cells.

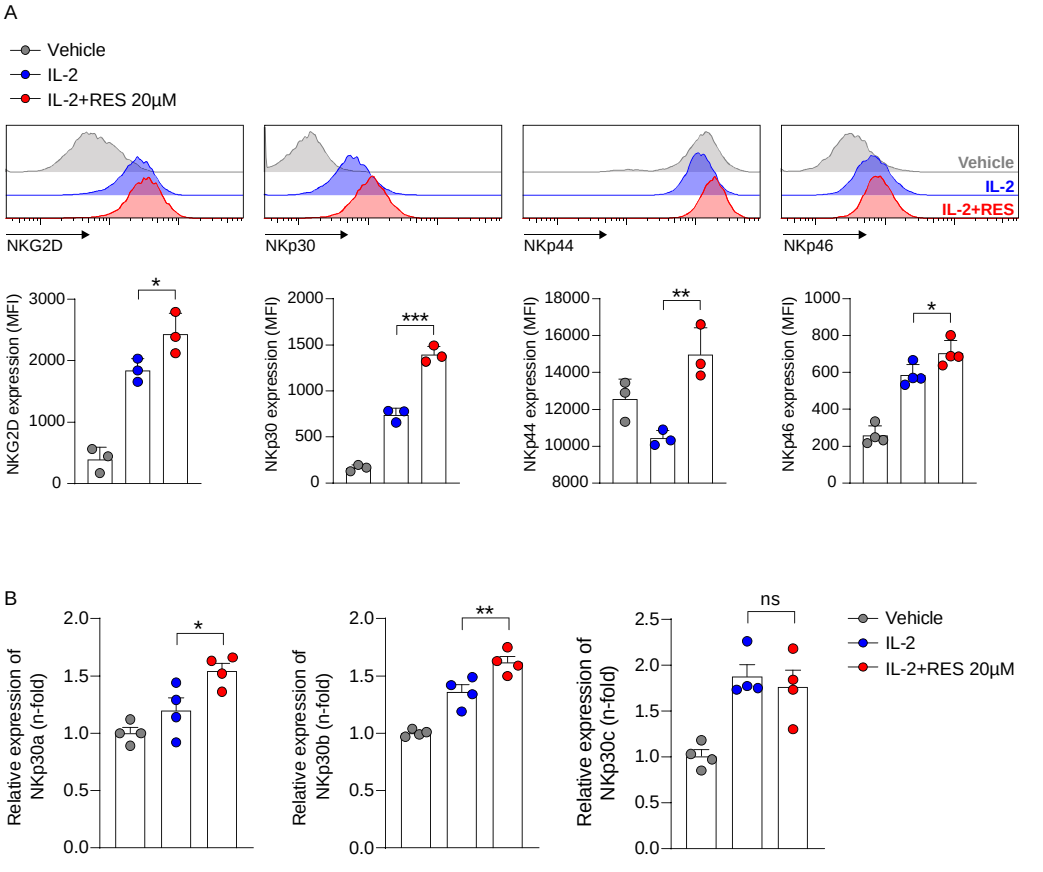


Figure 4. Effects of resveratrol on NK cell activity. (A) Expression of NCRs and NKG2D was measured by flow cytometry. NK92 cells were treated with 20 μM resveratrol for 36 hrs. Data are shown as mean ± SEM of 3 independent experiments. (B) The mRNA levels of NKp30 isoforms in NK92 cells after treatment with resveratrol for 6 hrs were measured by real-time PCR and calculated by the ΔΔCT method. GAPDH was used as an internal control. Data are shown as mean ± SEM of 4 independent experiments. Asterisks indicate statistical significance by one-way ANOVA: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns: not significant ($p>0.05$).

5. Effects of derivatives and metabolites of resveratrol

Resveratrol is currently marketed as a dietary supplement. Triacetyl resveratrol, as a derivative of resveratrol, is easily converted to resveratrol by esterase activity and exhibits similar bioactivity as resveratrol in cell cultures. Once resveratrol absorbed, it is rapidly metabolized by conjugation to a hydroxyl group, glucuronic acid, and/or sulfate. The sites of glucuronidation in resveratrol in the 3- and 4′-positions have been firmly established after synthesis of the metabolites. Similarly, the major site of sulfate conjugation, in the 4′-position, was also established by synthesis (Figure 5A).[49] Therefore, an investigation was conducted into whether the derivative or any of the metabolites of resveratrol could activate NK cells better than resveratrol itself. First, the effects of these compounds on NK cells proliferation were investigated. Triacetyl resveratrol, a derivative of resveratrol, slightly inhibited cell proliferation, but the other compounds did not (Figure 5B). Next, IFN-γ secretion in NK cells was measured after treatment with the resveratrol derivative and metabolites. As shown in Figure 5C, the derivative and most of the metabolites did not affect IFN-γ secretion. Triacetyl resveratrol and resveratrol trisulfate increased IFN-γ secretion compared to that observed in IL-2 alone-treated cells, but their effect was weaker than that of resveratrol. Finally, the effects of triacetyl resveratrol and resveratrol trisulfate on NK cytotoxicity were examined using K562 target cells and the Calcein-AM method. As shown in Figure 5D, after treatment with resveratrol itself, NK cytotoxicity was increased by 20% at E:T ratios of 1:1 compared to that of IL-2 treatment alone. After triacetyl resveratrol treatment, NK cytotoxicity was increased by 11.4% at E:T ratios of 1:1, but this was

not statistically significant. Unlike resveratrol and triacetyl resveratrol, resveratrol trisulfate did not affect NK cytotoxicity. These results indicate that the intact form of resveratrol is the most effective for activating NK cells.

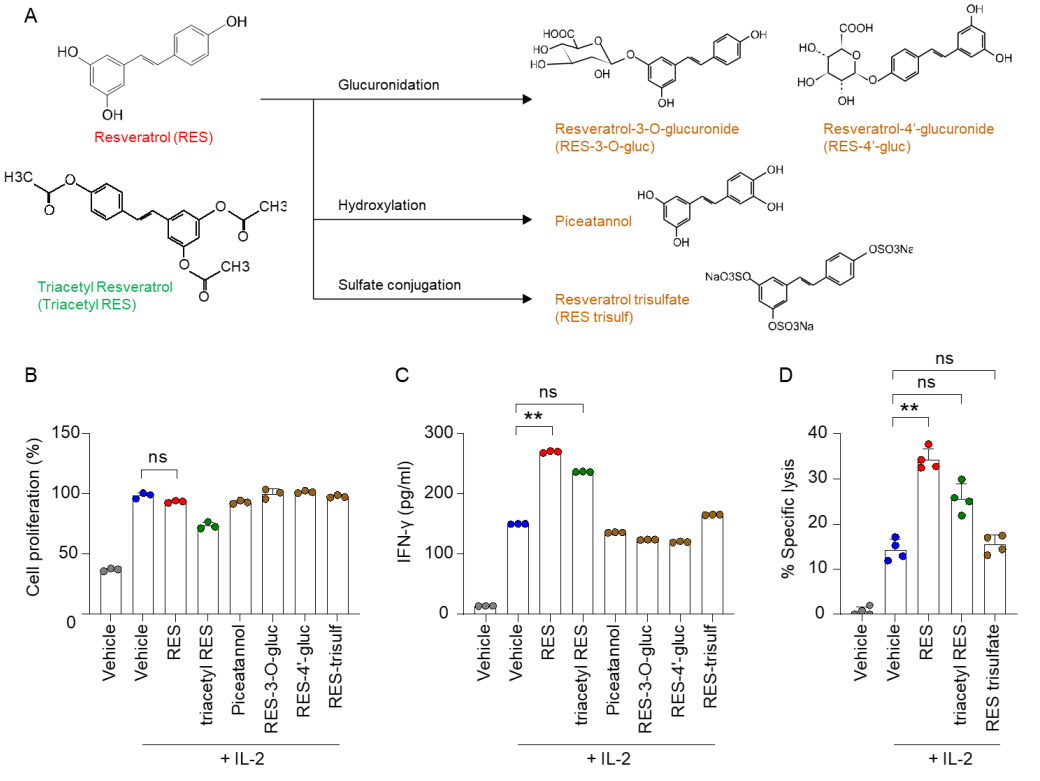


Figure 5. Effects of derivatives and metabolites of resveratrol. (A) Metabolism of resveratrol and structures of resveratrol, triacetyl resveratrol (derivative of resveratrol), and metabolites of resveratrol. Resveratrol is metabolized by hydroxylation or glucuronidation, or sulfate conjugation to produce combined forms of sulfate and glucuronide. (B-C) Cell proliferation and IFN-γ secretion in NK92 cells after indicated treatments at 20 μM (with 5 ng/ml of IL-2) for 36 hrs were determined by CCK-8 and ELISA. Data are shown as mean ± SEM of 3 independent experiments. (D) NK cells cytotoxicity was determined using calcein-AM. NK92 effector cells were treated with 20 μM resveratrol or its metabolites for 36 hrs, followed by incubation with K562 target cells at a 1:1 ratio for 4 hrs. Data are shown as mean ± SEM of 4 independent experiments. Asterisks indicate statistical significance by one-way ANOVA: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns: not significant ($p>0.05$).

6. Ex vivo effects of resveratrol in human and mouse whole blood

To investigate the effect of resveratrol on human and mouse primary NK cell activity, NK activity in whole blood using the NK Vue Kit was measured. After resveratrol treatment with activator, human NK cell activity increased by about 25% compared to treatment with activator alone (Figure 6A). Likewise, after resveratrol treatment with activator, tumor-naïve mouse NK cell activity increased by about 27.2% compared to treatment with activator alone (Figure 6B). Also, NK cell activity was measured in tumor-bearing mouse models. Even in the presence of an activator, NK cell activity was significantly lower in the tumor-bearing mice than in the tumor-naïve group (Figure 6B). However, after resveratrol treatment in the presence of activator, NK cell activity increased by about 27.3% compared to treatment with activator alone in the tumor-bearing mice (Figure 6B). Further, PBMCs were isolated from whole blood and treated with resveratrol for 24 hrs in the presence of IL-2, and then stimulated by K562 target cells (Figure 6C). Intriguingly, compared with IL-2 treated cells, it was found that resveratrol significantly enhanced degranulation in $CD56^{dim}CD16^{+}$ human primary NK cells known for their cytolytic activity (Figure 6C).[50] Thus, these results indicate that resveratrol has the potential to activate human primary NK cells and suggest that resveratrol could be used in cancer therapy by activating NK cells.

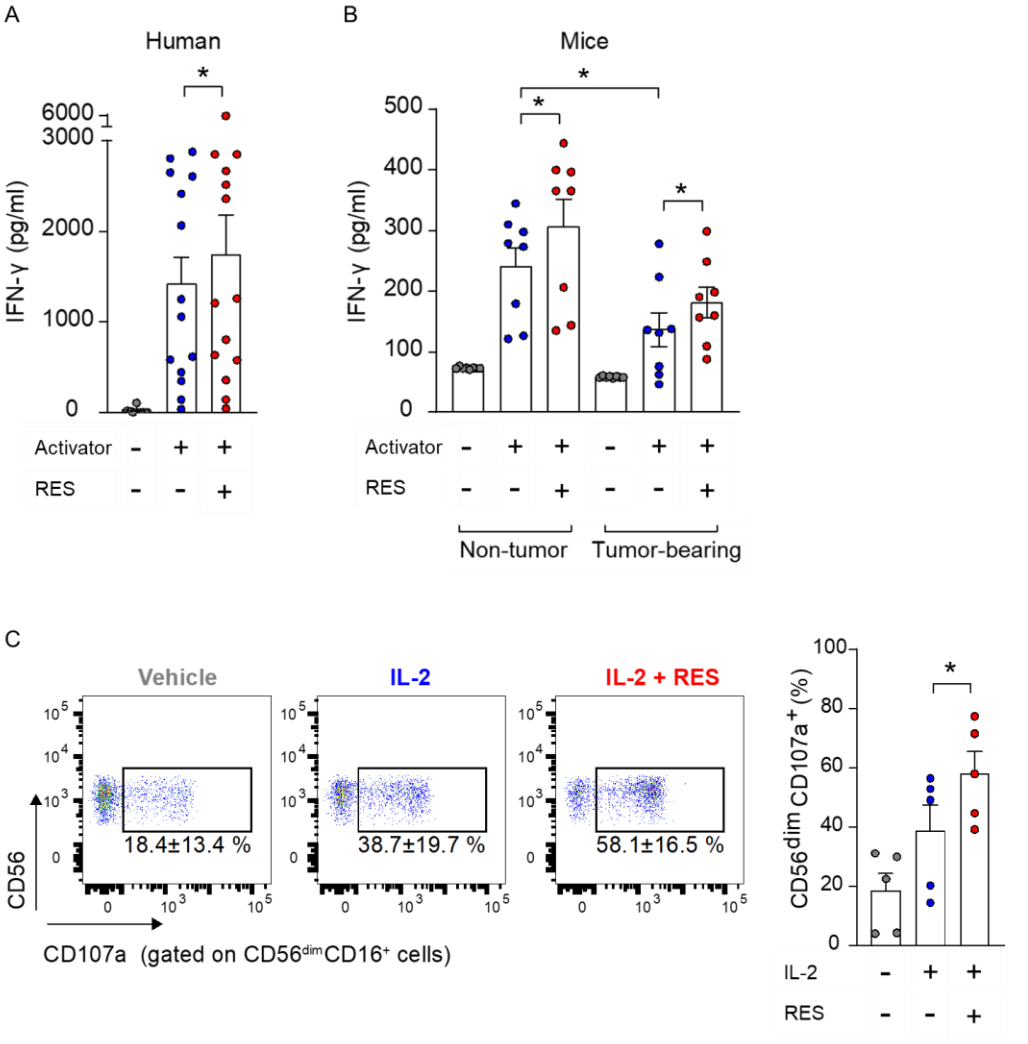
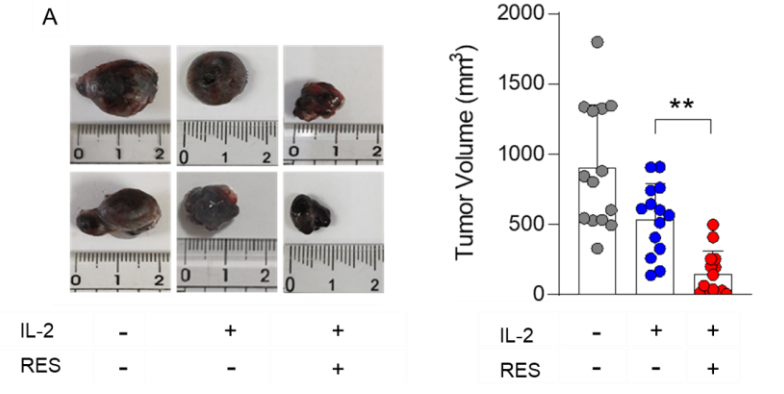
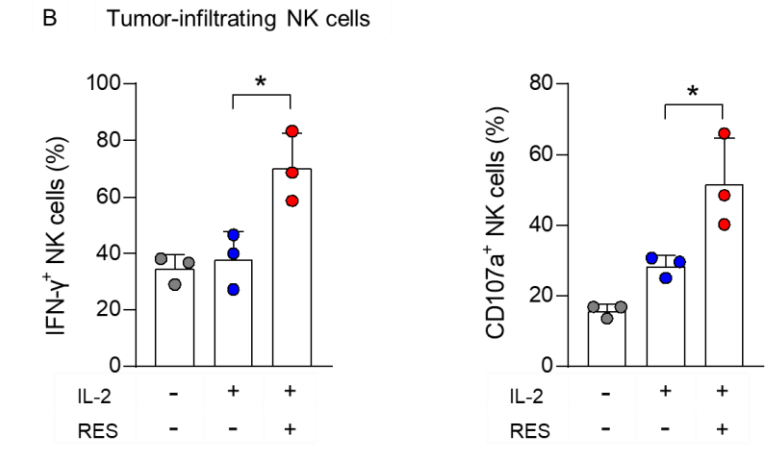


Figure 6. Ex vivo effects of resveratrol in human and mouse whole blood. (A) For the human NK cell activity assay, 1 ml of whole blood was incubated with or without activator and resveratrol for 24 hrs at 37°C. (B) For the mouse NK cell activity assay, 100 μl of whole blood was incubated with or without 30 μl activator and resveratrol for 24 hrs at 37°C (tumor-naïve or tumor-bearing mouse model, n=8). The supernatant was collected, and the released IFN-γ was measured using a NK Vue Kit. (C) CD107a expression of primary NK cells was measured by flow cytometry. PBMCs were treated with 20 μM resveratrol for 24 hrs in the presence of IL-2, followed by co-culturing with K562 target cells at a 5:1 (E:T) ratio for 4 hrs (n=5). Asterisks indicate statistical significance by Dunn's multiple comparison test: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns: not significant ($p>0.05$).

7. Anti-cancer effect of resveratrol in mice

The in vivo efficacy of resveratrol administration in melanoma mouse models was examined. In vivo activation of NK cells by resveratrol and its anti-cancer effects were assessed by 2 different methods. First, 18 days after s.c. injection of B16F10 cells, tumor volumes were measured after dissection. As shown in Figure 7A, administration of resveratrol (0.5 mg/kg) in the presence of IL-2 significantly inhibited tumor growth compared to the administration of IL-2 alone. Quantitative analysis showed that administration of IL-2 inhibited tumor growth compared to that in vehicle control mice. Furthermore, administration of resveratrol in the presence of IL-2 significantly inhibited tumor growth compared to the administration of IL-2 alone. Treatment of resveratrol together with IL-2 significantly inhibited tumor growth (Figure 7A) accompanied with robust activation of NK cells in tumors (Figure 7B) as well as draining lymph nodes (Figure 7C) compared to the vehicle or IL-2 treated group.





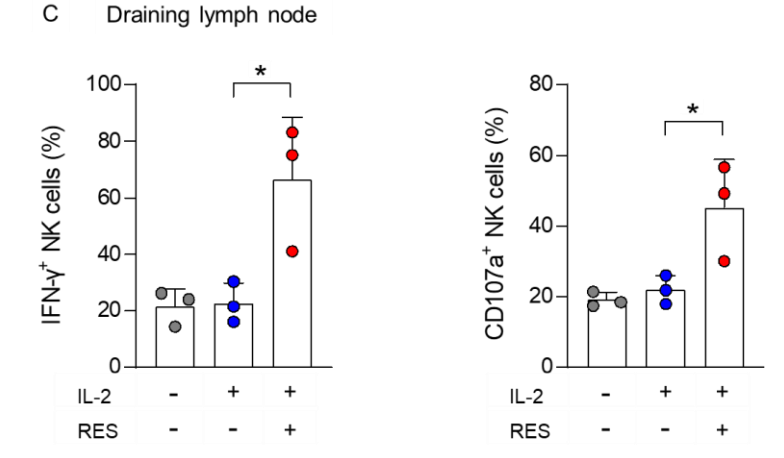


Figure 7. Anti-cancer effect of resveratrol in mice. B16F10 cells (5×10^5) in 0.2 ml PBS were injected subcutaneously into C57BL/6 mice on day 0 (n=14). (A) The tumor volume was measured after dissection on day 18 after injection of melanoma cells. The activity of NK cells isolated from (B) tumor and (C) draining lymph nodes was evaluated by flow cytometry. To evaluate the activity of resveratrol, mice were intravenously injected a total of 6 times, on days –2, 0, 2, 4, 6, and 8. Asterisks indicate statistical significance by one-way ANOVA: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns: not significant ($p>0.05$).

8. Anti-metastatic effects of resveratrol in mice

Next, it was investigated whether resveratrol affects tumor metastasis in the lung metastatic model as well. B16F10 cells were i.v. injected in mice; after 18 days, the lungs were extracted, and the melanoma foci on lung surfaces were counted. As shown in Figure 8A, the number of melanoma foci decreased in mice treated with resveratrol. Furthermore, IFN-γ production and degranulation level were significantly enhanced in the lung residual NK cells by the treatment of resveratrol compared with the vehicle or IL-2 treated group (Figure 8B). These results suggest that resveratrol may prohibit tumor growth and metastasis by activating NK cells.

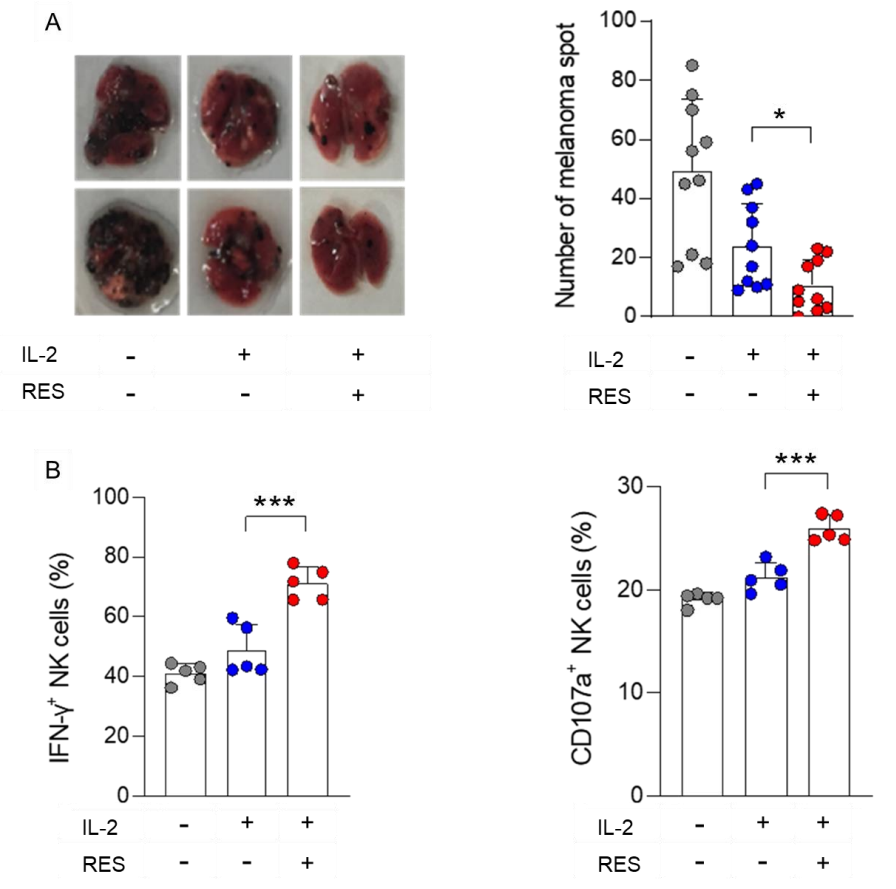


Figure 8. Anti-metastatic effects of resveratrol in mice. B16F10 cells (1×10^5) in 0.1 ml PBS were injected into the tail veins of C57BL/6 mice on day 0 (n=10). (A) The melanoma foci on the lung surface were counted macroscopically 18 days after injection of melanoma cells. (B) The activity of NK cells isolated from lung was evaluated by flow cytometry. To evaluate the activity of resveratrol, mice were intravenously injected a total of 6 times, on days –2, 0, 2, 4, 6, and 8. Asterisks indicate statistical significance by one-way ANOVA: *p<0.05, **p<0.01, ***p<0.001, ns: not significant (p>0.05).

9. NK cell-dependent anti-cancer effects of resveratrol

Finally, to clarify the intrinsic roles of NK cells on the anti-tumoral effects of resveratrol, mice were injected with tumors and treated with vehicle, resveratrol or resveratrol plus anti-NK1.1 to deplete NK cells in vivo. Intriguingly, as shown in Figure 9, the anti-tumoral effects of resveratrol were almost completely abrogated by the depletion of NK cells. Consistent with previous results, resveratrol treatment with isotype antibody significantly reduced tumor growth. However, the anti-tumoral effects of resveratrol were abrogated by the depletion of NK cells. Altogether, these results indicate the pivotal roles of NK cells in resveratrol dependent anti-tumoral effects.

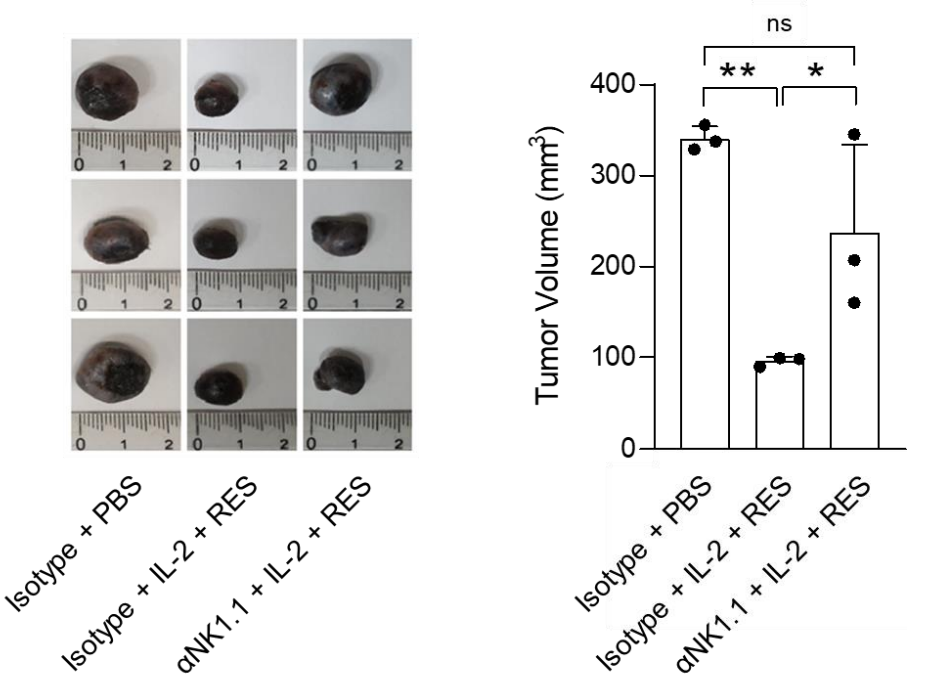
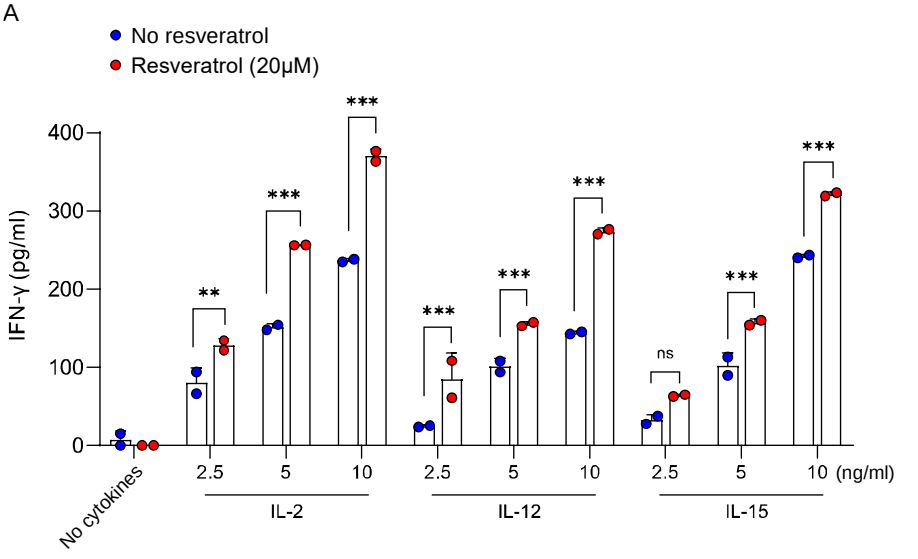


Figure 9. NK cell-dependent anti-cancer effects of resveratrol. To deplete NK cells, 25 μg anti-NK1.1 monoclonal antibody (PK136) or control mouse IgG2a were injected intraperitoneal (i.p.) on days -3, 2, and 8. B16F10 cells (5×10^5 cells) in 0.2 ml of PBS were injected subcutaneously into the right flanks of mice on day 0. The tumor volume was measured after dissection on day 18 after injection of melanoma cells. To evaluate the activity of resveratrol, mice were intravenously injected a total of 6 times, on days –2, 0, 2, 4, 6, and 8. Asterisks indicate statistical significance by one-way ANOVA: *p<0.05, **p<0.01, ***p<0.001, ns: not significant (p>0.05).

Part 2. Effects of resveratrol on NK cell activity through activation of Akt-mTOR mediated c-Myb transcription factor

1. Synergistic effect of resveratrol on NK cell activation in combination with IL-2

To identify whether resveratrol has a synergistic effect with cytokines that mediate NK cell activation, IFN-γ secretion and NK cytotoxicity were measured after treatment with resveratrol and cytokines. IL-2, IL-12, and IL-15 are known to induce IFN-γ secretion and play an important role in the activation of NK cells.[51,52] In part 1, it was shown that resveratrol activates NK cells in a synergistic manner with IL-2. To identify whether resveratrol has a synergistic effect with other cytokines that mediate NK cell activation, IFN-γ secretion and NK cytotoxicity were first measured after treatment with resveratrol and IL-2, IL-12, or IL-15. Among the cytokines, IL-2 produces the most IFN-γ secretion at the same concentration compared with other cytokines when they were co-treated with resveratrol (Figure 10A). In addition, it was found that treatment of resveratrol in combination with 5 ng/ml of IL-2 was as effective as treatment with 10 ng/ml of IL-2 alone (Figure 10A). Next, it was investigated whether resveratrol also has a synergistic effect with activating cytokines on the cytotoxicity of NK cells. As shown in Figure 10B, resveratrol showed a synergistic effect on the enhancement of NK cytolytic activity only in combination with IL-2. These results suggest that resveratrol may activate a factor downstream of the IL-2 signaling pathway.



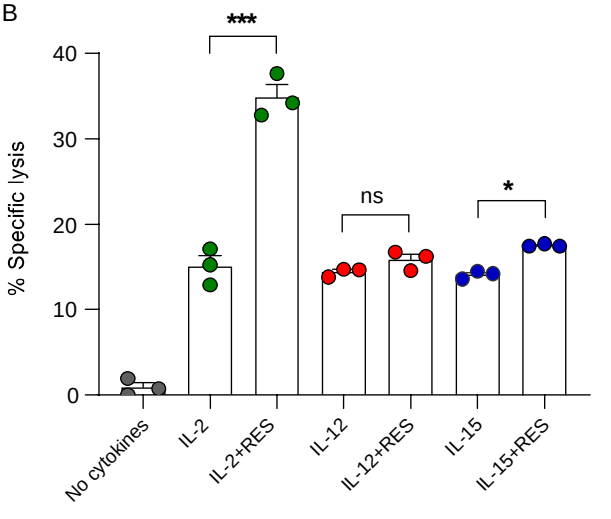
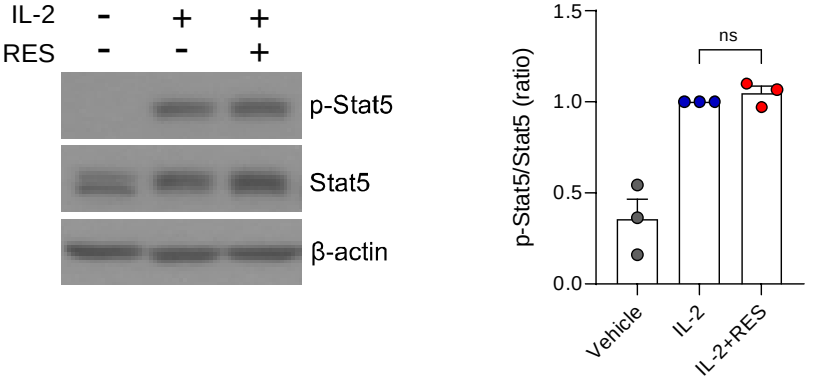


Figure 10. Synergistic effect of resveratrol on NK cell activation in combination with IL-2. (A) IFN-γ secretion in NK92 cells after indicated treatments at various different concentrations for 48 hrs were determined by ELISA. Data are shown as mean ± SEM of 2 independent experiments. (B) NK cell cytotoxicity was determined using calcein-AM. NK92 effector cells were treated with each cytokine (IL-2; 5 ng/ml, IL-12 and IL-15; 10 ng/ml) with or without 20 μM resveratrol for 36 hrs, followed by incubation with K562 target cells at a 1:1 ratio for 4 hrs. Data are shown as mean ± SEM of 3 independent experiments. Asterisks indicate statistical significance by one-way ANOVA: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns: not significant ($p>0.05$).

2. Regulation of Akt and mTORC2 signaling pathways by resveratrol

IL-2 signaling is propagated following receptor-ligand engagement, controlling recruitment of JAK3 and activation of AKT, ERK1/2 and a transcriptionally active STAT5.[53,54] Based on this information, an investigation was conducted into which molecule(s) is regulated by resveratrol among these IL-2 signaling mediated proteins. As shown in Figure 11, it was found that resveratrol increased the phosphorylation of Akt on Ser473 which is essential for full activation, but had no effect on the phosphorylation of Stat5 and ERK. These results suggest that resveratrol activates the Akt signaling pathway, but not the Stat5 and ERK signaling pathways.

A



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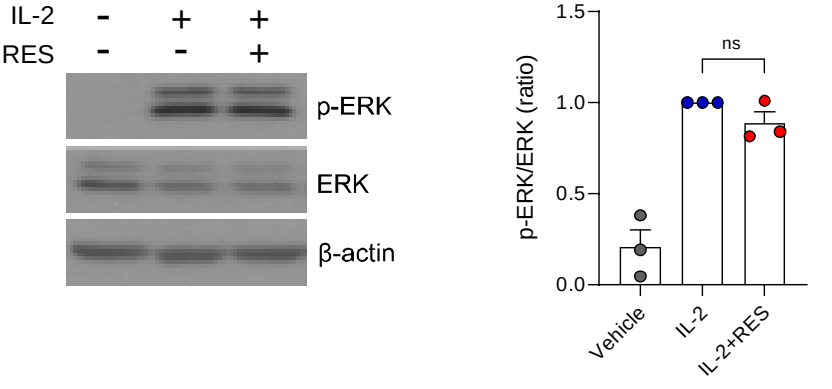

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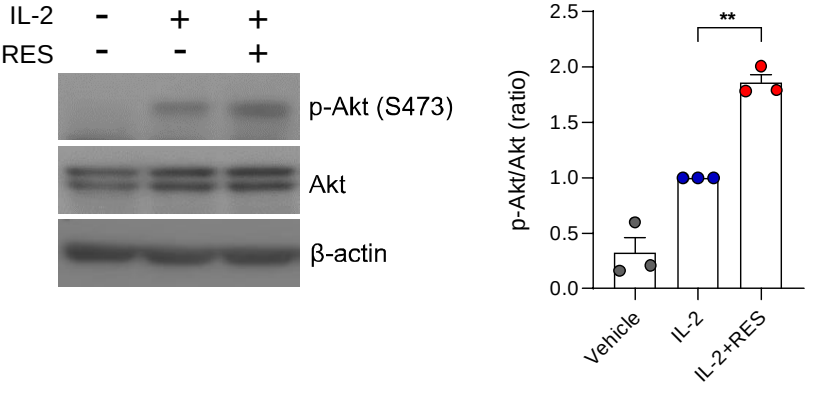
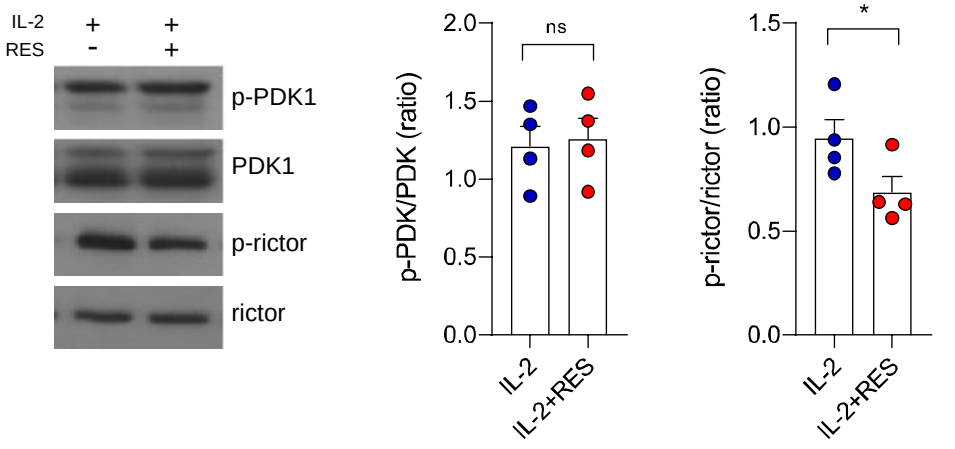


Figure 11. Regulation of Akt and mTORC2 signaling pathways by resveratrol. NK92 cells were deprived of IL-2 for 24 hrs then treated with IL-2 (5 ng/ml) with or without 20 μM resveratrol for 30 mins. Western blot was performed to visualize (A) Stat5 (pTyr694), (B) Erk (pThr202/Tyr204), and (C) Akt (pSer 473) expression as well as total form. Data are shown as mean ± SEM of 3 independent experiments. Asterisks indicate statistical significance by one-way ANOVA: *p<0.05, **p<0.01, ***p<0.001, ns: not significant (p>0.05).

3. Regulation of mTORC2 signaling pathway by resveratrol

Next, to find out how resveratrol activates Akt, measurements were taken of the expression levels of PDK1 and mTOR complex 2 (mTORC2), which are upstream kinases of Akt.[55] As shown in Figure 12A, PDK1 was not affected by resveratrol, whereas the phospho-rictor (a subunit of the mTORC2 complex) level decreased significantly. Previous studies have showed that phosphorylated rictor negatively regulates the mTORC2 protein complex as part of a negative feedback mechanism controlling Akt activity.[56] Consistent with this, resveratrol also appeared to inhibit PTEN and S6K1 activities which are known to downregulate mTORC2 signaling (Figure 12B). Taken all together, this data suggest that resveratrol activates Akt by regulating rictor phosphorylation, and mTORC2 via PTEN and S6K1.

A



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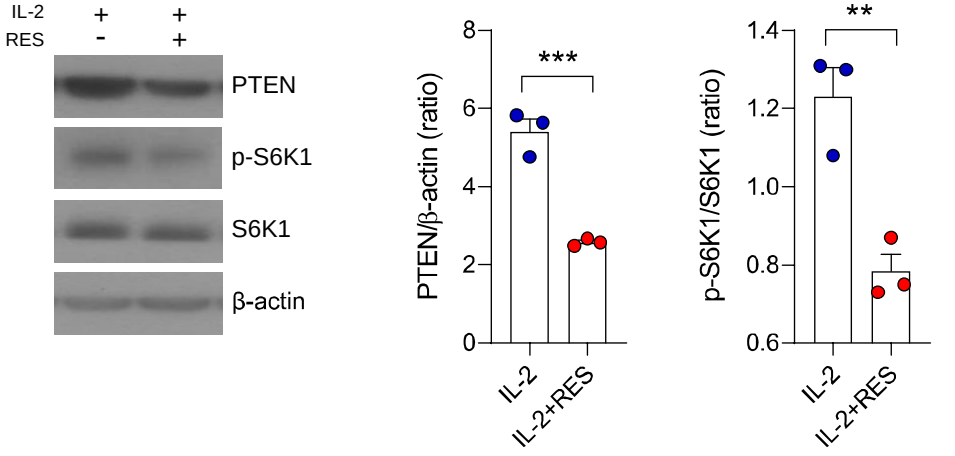


Figure 12. Regulation of mTORC2 signaling pathway by resveratrol. NK92 cells were deprived of IL-2 for 24 hrs then treated with IL-2 (5 ng/ml) with or without 20 μM resveratrol for 30 mins. (A) Western blot was performed to visualize PDK1 (pSer241) and rictor (pThr1135) expression as well as total form. Data are shown as mean ± SEM of 4 independent experiments. (B) Western blot was performed to visualize PTEN expression and S6K1 (pThr389) expression as well as total form. Data are shown as mean ± SEM of 3 independent experiments. Asterisks indicate statistical significance by Student's t-test: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns: not significant ($p>0.05$).

4. Effects of Akt and mTORC2 on NK cell activation by resveratrol

It was investigated whether NK cell activation is a direct effect of Akt and mTORC2 activation by resveratrol. For this purpose, IFN-γ secretion and the cytotoxicity of NK cells were measured after treatment with Akt inhibitor MK-2206. As shown in Figure 13A, IFN-γ secretion and cytotoxicity decreased by Akt inhibitor, but the inhibition was in substantial part overcome by resveratrol. These results may suggest that resveratrol may also activate NK cells in another way in addition to the Akt signaling pathway. Next, IFN-γ secretion and cytotoxicity were measured after treatment with mTOR inhibitor KU-0063794 (Figure 13B). As shown in Figure 13B, IFN-γ secretion and cytotoxicity decreased by mTOR inhibitor, but the inhibition was only a little overcome by resveratrol treatment. Interestingly, the mTOR inhibitor decreased the resveratrol induced NK cell activation more than that of the Akt inhibitor (Figure 13A and 13B). These results suggest that the resveratrol mediated NK cell activation is more dependent on the mTOR pathway than the Akt pathway.

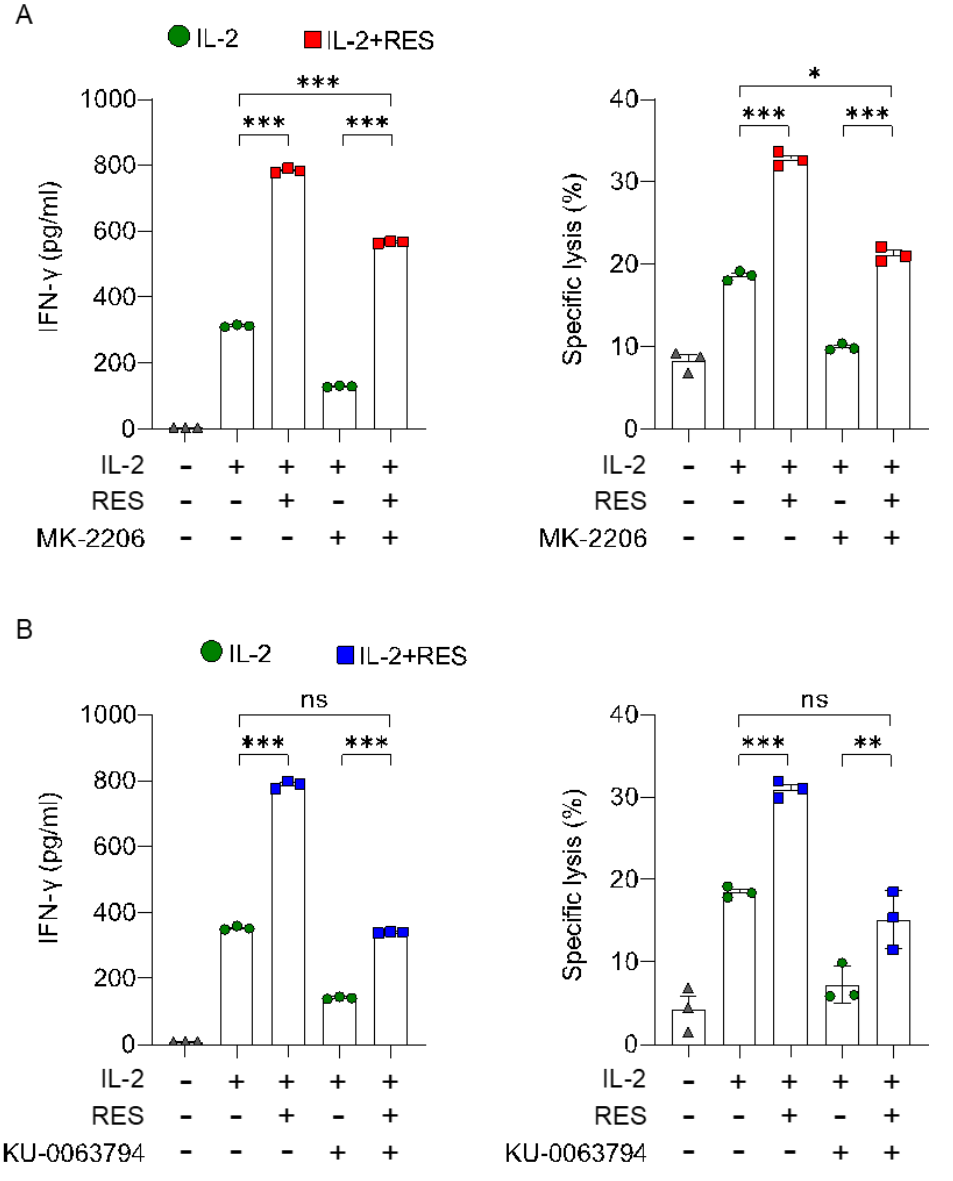


Figure 13. Effects of Akt and mTORC2 on NK cell activation by resveratrol. IFN-γ secretion in NK92 cells after indicated treatments for 48 hrs were determined by ELISA. NK cell cytotoxicity was determined using calcein-AM. NK92 effector cells were harvested after indicated treatments for 36 hrs, followed by incubation with K562 target cells at a 1:1 ratio for 4 hrs. (A) An Akt inhibitor (MK-2206) was added 30 mins before resveratrol treatment. (B) An mTOR inhibitor (KU-0063794) was added 30 mins before resveratrol treatment. Data are shown as mean ± SEM of 3 independent experiments. Asterisks indicate statistical significance by one-way ANOVA: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns: not significant ($p>0.05$).

5. Upregulation of Akt-related transcription factors in NK cells by resveratrol

Next, high-throughput analysis of transcription factors activation was performed using a DNA/Protein array to find transcription factors activated by resveratrol. As shown in Figure 14, the activities of 56 transcription factors in NK cells were screened by this method.

To select candidates among the 56 transcription factors whose activity was increased by resveratrol, first, candidates with a spot density of less than 4,000 were excluded. Next, transcription factors that exhibited a spot density increase of more than 2-fold by resveratrol compared with IL-2 alone treatment were selected. As a result, four transcription factors (CBF, c-Myb, NF-E2, and SP-1) were identified, and they were all found to be related to the Akt signaling pathway.[57-60] Resveratrol is already known to upregulate NF-E2, and it has been previously reported that c-Myb is a key factor in regulating NK cell activity.[61,62]

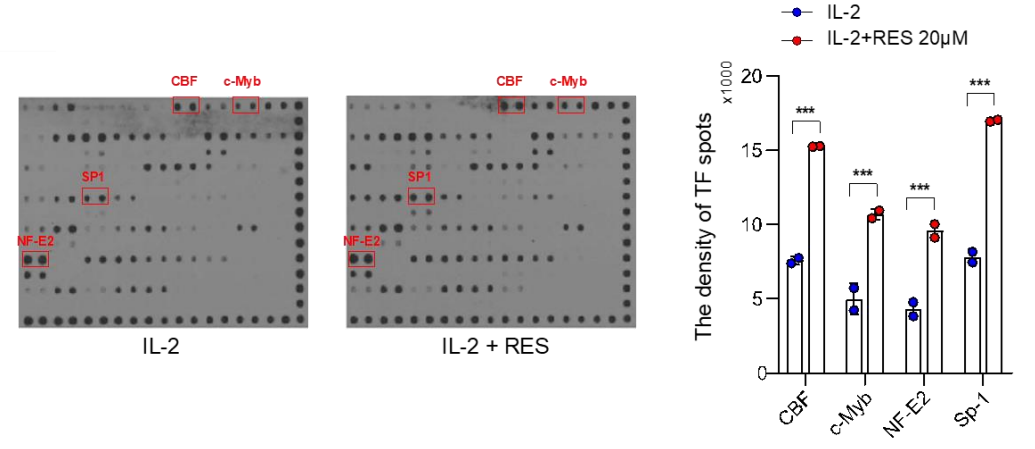


Figure 14. Upregulation of Akt-related transcription factors in NK cells by resveratrol. NK92 cells were deprived of IL-2 for 24 hrs then treated with IL-2 (5 ng/ml) with or without 20 μM resveratrol for 30 mins. Nuclear extracts were prepared and used for array assay. The array images were acquired and scanned. The quantitative result was displayed by measuring spot intensity with Image J software. Data are shown as mean ± SD of duplicate measurements. Asterisks indicate statistical significance by one-way ANOVA: *p<0.05, **p<0.01, ***p<0.001, ns: not significant (p>0.05).

6. Effect of resveratrol on c-Myb expression in NK cells

In this study, the effect of resveratrol on the expression of c-Myb was further investigated in more detail. As shown in Figure 15A and 15B, mRNA and protein expression levels of c-Myb appeared to increase by IL-2. Also as expected, resveratrol significantly increased the level of c-Myb expression compared with the IL-2 alone treated group. Next, it was investigated whether c-Myb activation by resveratrol is dependent on the Akt and mTOR pathways. As shown in Figure 15C, c-Myb expression decreased by Akt inhibitor, but the inhibition was overcome by resveratrol. Additionally, c-Myb expression decreased by mTOR inhibitor, but the inhibition was not completely overcome by resveratrol. Altogether, these results suggest that the mTOR pathway is more important in c-Myb upregulation by resveratrol than the Akt pathway.

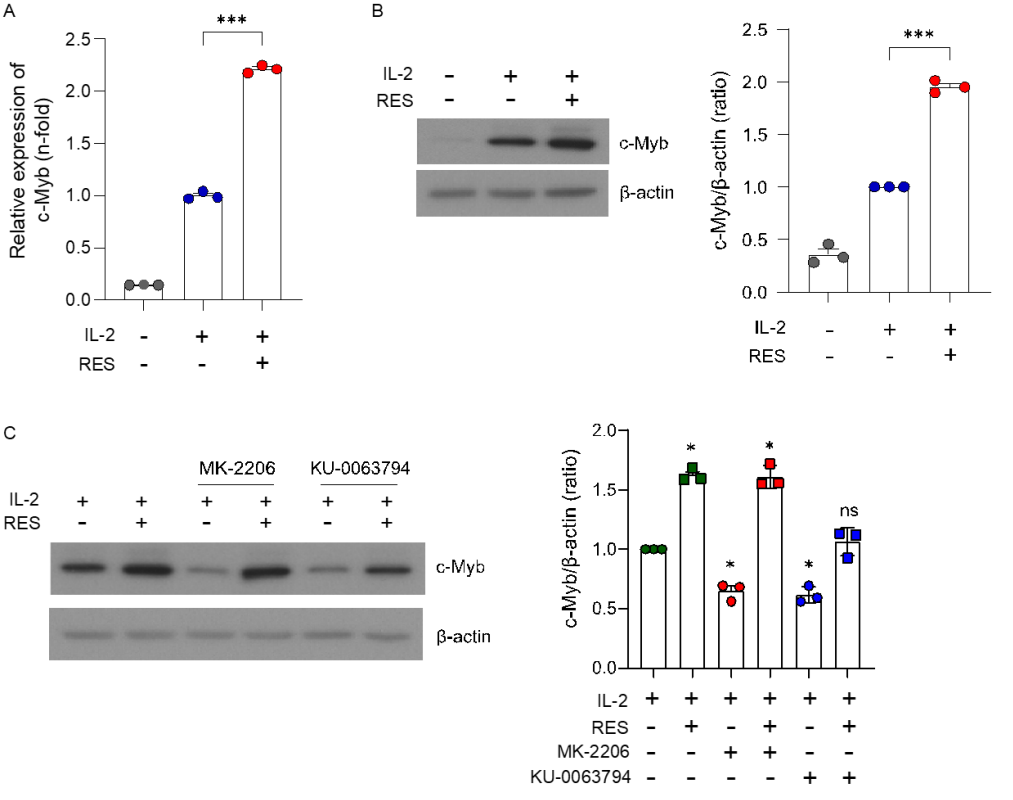
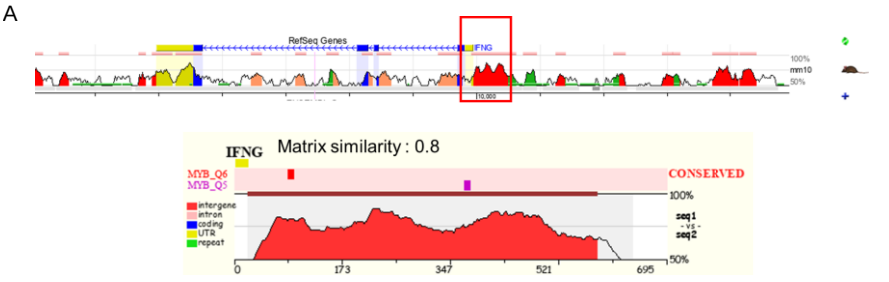
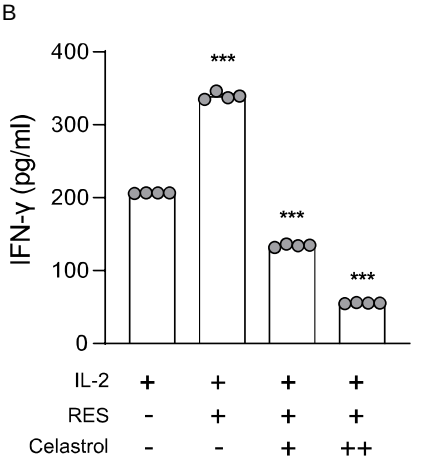


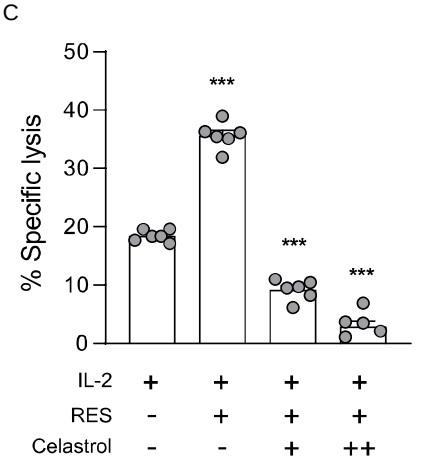
Figure 15. Effect of resveratrol on c-Myb expression in NK cells. (A) The mRNA levels of c-Myb in NK92 cells after treatment with resveratrol for 24 hrs were measured by real-time PCR and calculated by the ΔΔCT method. GAPDH was used as an internal control. (B) Western blot was performed to visualize c-Myb expression. Data are shown as mean ± SEM of 3 independent experiments. (C) Western blot was performed to visualize c-Myb expression after treatment of Akt inhibitor (MK-2206) and mTOR inhibitor (KU-0063794). Data are shown as mean ± SEM of 3 independent experiments. Asterisks indicate statistical significance by one-way ANOVA: *p<0.05, **p<0.01, ***p<0.001, ns: not significant (p>0.05).

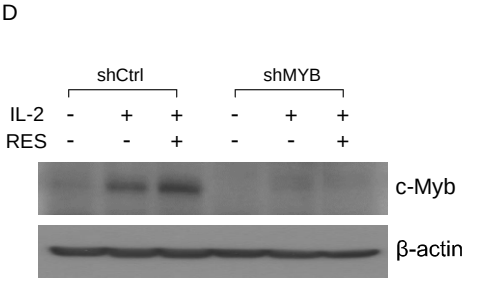
7. Effect of c-Myb on NK cell activation induced by resveratrol

Finally, the dependence of resveratrol on c-Myb to activate NK cells was investigated in more detail. To identify whether the c-Myb binding site exists within the IFN-γ promoter region, the ECR browser, a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes, was used. As shown in Figure 16A, it was predicted that the c-Myb binding site exists within the IFN-γ promoter region. Next, IFN-γ (Figure 16B) and cytolytic activity (Figure 16C) were measured after treatment with the c-Myb inhibitor celastrol to investigate whether resveratrol depends on c-Myb to activate NK cells. As shown in Figure 16B and 16C, the effects of resveratrol were completely abrogated upon the inhibition of c-Myb. Further confirmation that resveratrol depends on c-Myb was achieved by c-Myb knockdown studies in NK cells. c-Myb knockdown was confirmed by shRNA (Figure 16D), and the effect on NK cytotoxicity of resveratrol were completely abrogated by c-Myb knockdown (Figure 16E). Altogether, these results indicate that resveratrol activates NK cells dependent on c-Myb.









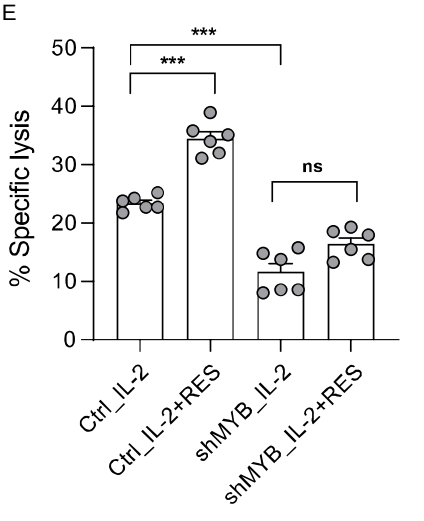


Figure 16. Effect of c-Myb on NK cell activation induced by resveratrol. (A) Annotation of conserved transcription factor binding sites underlying ECR Browser conservation plots were displayed using the 'Synteny/Alignments' link in the top menu. (B-C) A c-Myb inhibitor (celastrol) was added 30 mins before resveratrol treatment. (B) IFN-γ secretion in NK92 cells after indicated treatments for 36 hrs was determined by ELISA. Data are shown as mean ± SEM of 4 independent experiments. (C) NK cell cytotoxicity was determined using calcein-AM. NK92 effector cells were harvested after indicated treatments for 36 hrs, followed by incubation with K562 target cells at a 1:1 ratio for 4 hrs. Data are shown as mean ± SEM of 6 independent experiments. (D) Western blot was performed to visualize c-Myb expression after c-Myb knockdown using shRNA. (E) NK cell cytotoxicity was determined using calcein-AM. NK92 effector cells were harvested after indicated treatments for 36 hrs, followed by incubation with K562 target cells at a 1:1 ratio for 4 hrs. Data are shown as mean ± SEM of 6 independent experiments. Asterisks indicate statistical significance by one-way ANOVA: *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns: not significant ($p>0.05$).

IV. DISCUSSION

In part 1, it was found that resveratrol showed a synergistic effect in combination with IL-2 on NK cell activation. Resveratrol is a natural compound and antioxidant found in peanuts, soy, hop, and various fruits, especially grapes and red wines.[63,64] Resveratrol possesses various biological activities, including anti-tumor, anti-viral, anti-inflammation, anti-fungal, and antibacterial effects.[65-68] This study has demonstrated that resveratrol increases the expression of activating receptors on NK cells (Figure 4). Interestingly, it was found that resveratrol+IL-2 significantly increases NKp30 more than IL-2 alone. Especially NKp30a and NKp30b isoforms, which have immune-stimulatory roles, were significantly increased by resveratrol more than IL-2 alone. One of the proposed approaches for tumor therapy is enhancing tumor sensitivity to NK cells cytolysis by upregulating the NKp30 ligand B7-H6 on tumor cells using stress inducers.[69] Moreover, preclinical approaches involve increasing tumor sensitivity to NK cells by upregulating the expression of NKG2D ligands on cancer cells using HDAC inhibitors.[70] Enhancement of cell surface expression of NKG2D ligands and DR4/5 on various cancer cells by resveratrol increases TRAIL sensitivity and could facilitate NK cell-mediated killing of cancer cells.[71,72] However, there are no reports that resveratrol also increases B7-H6 expression on cancer cells. If resveratrol did induce the expression of B7-H6 on cancer cells, it would be a good candidate for an anti-cancer drug, acting as both an immune-stimulator for NK cells and a stress inducer for cancer cells.

In part 1, it was found that NK cells require IL-2 to proliferate, and resveratrol

treatment without IL-2 did not affect IFN-γ secretion or NK cytotoxicity in vitro (Figure 2). IL-2 is capable of activating NK cells and is used as an anti-cancer drug in immunotherapy.[73] According to a recent report, resveratrol has shown promising results in adjuvant therapy.[63] Resveratrol in combination with 5-fluoruracyl or cisplatin has exhibited synergistic effects in the induction of apoptosis in cancer cells in vitro and in vivo.[74,75] Therefore, resveratrol can be expected to exert a greater effect in combination with IL-2.

Resveratrol shows a concentration-dependent biphasic effect.[76] Low doses of resveratrol reportedly activate NK cells, while high doses reportedly promote cell apoptosis via the caspase signaling pathway.[76] In one study, NK cells killing activity was enhanced at low concentrations of resveratrol ranging from 0.33 μM to 5.48 μM, with maximum activity at 1.31 μM; however, NK cells killing activity was inhibited at higher concentrations of 21.92 μM and 87.68 μM resveratrol.[44] In another study by Lu and Chen,[45] 12.5 μM resveratrol induced the highest killing activity of NK cells. In this study, resveratrol did not significantly affect NK cells proliferation in vitro, but it did increase IFN-γ secretion in a dose-dependent manner up to a concentration of 20 μM. It was also found that resveratrol increased IFN-γ secretion ex vivo at a concentration of 20 μM and did not affect survival when injected into mice at 0.5 mg/kg (data not shown).

Resveratrol is currently being sold as a dietary supplement. Resveratrol is first detectable in plasma 15 mins after oral administration, reaching its peak concentration after 30 mins, and it is quickly metabolized in the intestine and liver into glucuronide and sulfate conjugates.[77] According to a study on the absorption of wine-related

polyphenols, the highest serum level of total resveratrol (free and conjugated) was recorded after consumption of 25 mg trans-resveratrol per 70 kg body weight in 3 different matrices (wine, grape juice, and vegetable juice).[78] Free trans-resveratrol accounted for 1.7–1.9% of the peak total serum concentration. This is considerably lower than the concentrations tested in vitro showing beneficial effects. Moreover, resveratrol is rapidly metabolized in the intestine by phase II enzymes, so it is very difficult to reach pharmacologically effective systemic plasma concentrations, which might significantly restrict the bioavailability of resveratrol.[79,80] This study showed that IFN-γ secretion increased upon treatment with intact resveratrol but not with its metabolites (Figure 5). Accordingly, resveratrol would be more effective when administered by injection rather than by diet.

In part 2, it was found that resveratrol showed a synergistic effect in combination with IL-2, IL-12, and IL-15 on IFN-γ secretion, but a synergistic effect with only IL-2 on NK cytolytic activity (Figure 10). Furthermore, it was demonstrated that resveratrol activates Akt by regulating mTORC2 via PTEN and S6K1 (Figure 11 and 12). Also, it was found that resveratrol mediated NK cell activation is more dependent on the mTOR pathway than the Akt pathway, and resveratrol upregulates c-Myb expression, which is a key regulator of NK cell activity (Figure 13-16). mTORC2 directly activates PKC and SGK, and the SGK gene families which are known to be similar to the MYB gene family.[81] Interestingly, SGK1 is known to a factor downstream of mTORC2 and regulate Th1 and Th2 differentiation.[82] Therefore, it would be interesting to confirm whether mTORC2 could also directly activate c-Myb, as in the case of SGK1.

MicroRNAs (miRs) are a highly conserved class of small, noncoding RNAs with

important regulatory functions in proliferation, differentiation, signal transduction, immune responses, and carcinogenesis.[83,84] It has been previously reported that miR-155 enhances IFN-γ production in NK cells and was upregulated by c-Myb transcription factor.[85,86] miR-150 is widely expressed in immune cells and has an important role in the development of lymphocytes and in hematopoietic malignancies.[87] Several studies have reported that miR-150 negatively regulates CD8 T cell memory formation and controls B cell differentiation by targeting the c-Myb transcription factor.[88,89] In this study, it was found that resveratrol activates NK cells by upregulating the expression of c-Myb (Figure 15). Therefore, it would be good to check whether resveratrol activates NK cells by stimulating miR-155 function as a result of upregulating c-Myb activity, or by inhibiting the miR-150 function.

Tumor progression usually leads to exhaustion of NK cells, thus limiting the anti-tumor potential of NK cells.[90] It has been reported that Tim-3 is involved in T cell exhaustion. Interestingly, Tim-3 is also expressed in NK cells.[91] Tim-3 expression in NK cells increases as the melanoma stage progresses and is higher in melanoma patients with poor prognostic factors.[91] Furthermore, Tim-3 blockade reverses the exhausted phenotype of NK cells.[91] These data open exciting avenues for new therapies targeting Tim-3 in tumor immunotherapy. It has previously been reported that IFN-γ secretion decreases in NK cells from tumor-bearing mice. This phenomenon could be due to the fact that those NK cells were already activated in vivo and at the time of sample collection those cells were in an exhaustion phase. It was also observed that resveratrol increased IFN-γ secretion in NK cells even from tumor-bearing mice, suggesting that resveratrol reverses the exhaustion of NK cells and overcomes the

limitation of the anti-tumor potential of NK cells due to exhaustion. Therefore, it would be good to check if resveratrol blocks Tim-3 or other exhaustion markers in NK cells from tumor-bearing mice.

This study has demonstrated that resveratrol enhances NK cell activity dependent on c-Myb. c-Myb is already known to enhance the function of CD8+ T cells, as well as NK cells. Altogether, it is expected that resveratrol could be a potential adjuvant for cancer immunotherapies, since it could enhance CD8+ T cell response as well as NK cell activity.

V. CONCLUSION

NK cells play a key role in anti-tumor and anti-viral infection in the absence of specific immunization. Recently researchers are actively studying to develop cancer therapies using NK cells to overcome the limitations of therapeutic efficacy. Resveratrol is a natural compound and antioxidant found in peanuts, soy, hop, and various fruits, especially grapes and red wines. Resveratrol is one of the well-studied polyphenols which has been shown to possess various potential health benefits, including anti-tumor, anti-viral, and antibacterial effects. Several studies have reported a synergistic effect of resveratrol in combination with doxorubicin in different breast cancer cell lines or apoptosis in colon cancer cell lines. Therefore, resveratrol has been suggested as a useful therapeutic adjuvant which has a strong synergism to maximize therapeutic efficacy for the treatment of cancer patients.

In part 1, it was demonstrated that resveratrol increases NK cytolytic activity by upregulating IFN-γ secretion and expression of activating receptors on NK cells. Interestingly, it was found that resveratrol+IL-2 significantly increases NKp30 more than IL-2 alone. Especially NKp30a and NKp30b isoforms, which have immune-stimulatory roles, were significantly increased by resveratrol more than IL-2 alone. One of the proposed approaches for tumor therapy is enhancing tumor sensitivity to NK cells cytolysis by upregulating the NKp30 ligand B7-H6 on tumor cells using stress inducers. Most importantly, administration of resveratrol effectively inhibited tumor growth and metastasis in vivo mouse model. As a result, this study suggests that resveratrol can be a potential adjuvant for cancer immunotherapies. However, the

mechanisms of how resveratrol activates NK cells are unclear.

In part 2, it was found that resveratrol has a synergistic effect in combination with IL-2. Resveratrol showed a synergistic effect in combination with cytokines such as IL-2, IL-12, and IL-15 on IFN-γ secretion, but a synergistic effect only with IL-2 on NK cytolytic activity. Among the IL-2 signaling mediated proteins, resveratrol activates the Akt signaling pathway by regulating rictor (a subunit of the mTORC2 complex) phosphorylation. Also, it was found that resveratrol regulates transcription factors related to the Akt signaling pathway. It had previously been reported that c-Myb transcription factor is a key factor in regulating NK cell activity, in this study, I found that resveratrol upregulated the c-Myb transcription factor. Finally, it was predicted that the c-Myb transcription factor binding site exists within the IFN-γ promoter region using the ECR browser. It was also found that the effects of resveratrol were completely abrogated on the inhibition of the c-Myb transcription factor. Altogether, these results indicate that resveratrol activates NK cells dependent on the c-Myb transcription factor.

REFERENCES

1. Mikulak J, Bruni E, Oriolo F, Di Vito C, Mavilio D. Hepatic Natural Killer Cells: Organ-Specific Sentinels of Liver Immune Homeostasis and Physiopathology. Front Immunol 2019;10:946.
2. Lodoen MB, Lanier LL. Natural killer cells as an initial defense against pathogens. Curr Opin Immunol 2006;18:391-8.
3. Cerwenka A, Lanier LL. Natural killer cells, viruses and cancer. Nat Rev Immunol 2001;1:41-9.
4. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol 2008;9:503-10.
5. Guillerey C, Huntington ND, Smyth MJ. Targeting natural killer cells in cancer immunotherapy. Nat Immunol 2016;17:1025-36.
6. Kim HR, Lee KH, Park SJ, Kim SY, Yang YK, Tae J, et al. Anti-cancer activity and mechanistic features of a NK cell activating molecule. Cancer Immunol Immunother 2009;58:1691-700.
7. Glasner A, Ghadially H, Gur C, Stanietsky N, Tsukerman P, Enk J, et al. Recognition and prevention of tumor metastasis by the NK receptor NKp46/NCR1. J Immunol 2012;188:2509-15.
8. Morvan MG, Lanier LL. NK cells and cancer: you can teach innate cells new tricks. Nat Rev Cancer 2016;16:7-19.
9. Zwirner NW, Domaica CI. Cytokine regulation of natural killer cell effector functions. Biofactors 2010;36:274-88.
10. Koch J, Steinle A, Watzl C, Mandelboim O. Activating natural cytotoxicity receptors of natural killer cells in cancer and infection. Trends Immunol 2013;34:182-91.
11. Abel AM, Yang C, Thakar MS, Malarkannan S. Natural Killer Cells: Development, Maturation, and Clinical Utilization. Front Immunol 2018;9:1869.
12. Smyth MJ, Hayakawa Y, Takeda K, Yagita H. New aspects of natural-killer-cell

surveillance and therapy of cancer. Nat Rev Cancer 2002;2:850-61.

13. Wang R, Jaw JJ, Stutzman NC, Zou Z, Sun PD. Natural killer cell-produced IFN-γ and TNF-α induce target cell cytolysis through up-regulation of ICAM-1. J Leukoc Biol 2012;91:299-309.
14. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. Science 2011;331:44-9.
15. Krieg C, Létourneau S, Pantaleo G, Boyman O. Improved IL-2 immunotherapy by selective stimulation of IL-2 receptors on lymphocytes and endothelial cells. Proc Natl Acad Sci U S A 2010;107:11906-11.
16. Dempke WCM, Fenchel K, Uciechowski P, Dale SP. Second- and third-generation drugs for immuno-oncology treatment-The more the better? Eur J Cancer 2017;74:55-72.
17. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nat Rev Immunol 2010;10:317-27.
18. Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: from T cell basic science to clinical practice. Nat Rev Immunol 2020:1-18.
19. Wculek SK, Cueto FJ, Mujal AM, Melero I, Krummel MF, Sancho D. Dendritic cells in cancer immunology and immunotherapy. Nat Rev Immunol 2020;20:7-24.
20. Shimasaki N, Jain A, Campana D. NK cells for cancer immunotherapy. Nat Rev Drug Discov 2020;19:200-18.
21. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. Cell 2017;168:707-23.
22. Chiossone L, Dumas PY, Vienne M, Vivier E. Natural killer cells and other innate lymphoid cells in cancer. Nat Rev Immunol 2018;18:671-88.
23. Van Schandevyl S, Kerre T. Chimeric antigen receptor T-cell therapy: design improvements and therapeutic strategies in cancer treatment. Acta Clin Belg 2020;75:26-32.
24. Nowicki TS, Hu-Lieskovan S, Ribas A. Mechanisms of Resistance to PD-1 and

PD-L1 Blockade. Cancer J 2018;24:47-53.

25. van Elsas MJ, van Hall T, van der Burg SH. Future Challenges in Cancer Resistance to Immunotherapy. Cancers (Basel) 2020;12.
26. Darvin P, Toor SM, Sasidharan Nair V, Elkord E. Immune checkpoint inhibitors: recent progress and potential biomarkers. Exp Mol Med 2018;50:1-11.
27. Wigginton JM, Wiltrout RH. IL-12/IL-2 combination cytokine therapy for solid tumours: translation from bench to bedside. Expert Opin Biol Ther 2002;2:513-24.
28. Atzpodien J, Kirchner H, Illiger HJ, Metzner B, Ukena D, Schott H, et al. IL-2 in combination with IFN- alpha and 5-FU versus tamoxifen in metastatic renal cell carcinoma: long-term results of a controlled randomized clinical trial. Br J Cancer 2001;85:1130-6.
29. Danielli R, Patuzzo R, Di Giacomo AM, Gallino G, Maurichi A, Di Florio A, et al. Intralesional administration of L19-IL2/L19-TNF in stage III or stage IVM1a melanoma patients: results of a phase II study. Cancer Immunol Immunother 2015;64:999-1009.
30. Gladkov O, Ramlau R, Serwatowski P, Milanowski J, Tomeczko J, Komarnitsky PB, et al. Cyclophosphamide and tucotuzumab (huKS-IL2) following first-line chemotherapy in responding patients with extensive-disease small-cell lung cancer. Anticancer Drugs 2015;26:1061-8.
31. Buzio C, Andrulli S, Santi R, Pavone L, Passalacqua R, Potenzoni D, et al. Long-term immunotherapy with low-dose interleukin-2 and interferon-alpha in the treatment of patients with advanced renal cell carcinoma. Cancer 2001;92:2286-96.
32. Szczepanski MJ, Szajnik M, Welsh A, Foon KA, Whiteside TL, Boyiadzis M. Interleukin-15 enhances natural killer cell cytotoxicity in patients with acute myeloid leukemia by upregulating the activating NK cell receptors. Cancer Immunol Immunother 2010;59:73-9.
33. Zhu EF, Gai SA, Opel CF, Kwan BH, Surana R, Mihm MC, et al. Synergistic innate and adaptive immune response to combination immunotherapy with

anti-tumor antigen antibodies and extended serum half-life IL-2. Cancer Cell 2015;27:489-501.

34. Khuda-Bukhsh AR, Das S, Saha SK. Molecular approaches toward targeted cancer prevention with some food plants and their products: inflammatory and other signal pathways. Nutr Cancer 2014;66:194-205.
35. Khan N, Afaq F, Mukhtar H. Cancer chemoprevention through dietary antioxidants: progress and promise. Antioxid Redox Signal 2008;10:475-510.
36. Sinha D, Biswas J, Sung B, Aggarwal BB, Bishayee A. Chemopreventive and chemotherapeutic potential of curcumin in breast cancer. Curr Drug Targets 2012;13:1799-819.
37. Han G, Xia J, Gao J, Inagaki Y, Tang W, Kokudo N. Anti-tumor effects and cellular mechanisms of resveratrol. Drug Discov Ther 2015;9:1-12.
38. de Sá Coutinho D, Pacheco MT, Frozza RL, Bernardi A. Anti-Inflammatory Effects of Resveratrol: Mechanistic Insights. Int J Mol Sci 2018;19.
39. Filardo S, Di Pietro M, Mastromarino P, Sessa R. Therapeutic potential of resveratrol against emerging respiratory viral infections. Pharmacol Ther 2020;214:107613.
40. Ma DSL, Tan LT, Chan KG, Yap WH, Pusparajah P, Chuah LH, et al. Resveratrol-Potential Antibacterial Agent against Foodborne Pathogens. Front Pharmacol 2018;9:102.
41. Kiziltepe U, Turan NN, Han U, Ulus AT, Akar F. Resveratrol, a red wine polyphenol, protects spinal cord from ischemia-reperfusion injury. J Vasc Surg 2004;40:138-45.
42. Jin X, Wei Y, Liu Y, Lu X, Ding F, Wang J, et al. Resveratrol promotes sensitization to Doxorubicin by inhibiting epithelial-mesenchymal transition and modulating SIRT1/β-catenin signaling pathway in breast cancer. Cancer Med 2019;8:1246-57.
43. Feng M, Zhong LX, Zhan ZY, Huang ZH, Xiong JP. Resveratrol Treatment Inhibits Proliferation of and Induces Apoptosis in Human Colon Cancer Cells. Med Sci Monit 2016;22:1101-8.

44. Falchetti R, Fuggetta MP, Lanzilli G, Tricarico M, Ravagnan G. Effects of resveratrol on human immune cell function. Life Sci 2001;70:81-96.
45. Lu CC, Chen JK. Resveratrol enhances perforin expression and NK cell cytotoxicity through NKG2D-dependent pathways. J Cell Physiol 2010;223:343-51.
46. Alter G, Malenfant JM, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. J Immunol Methods 2004;294:15-22.
47. Lozzio BB, Lozzio CB. Properties and usefulness of the original K-562 human myelogenous leukemia cell line. Leuk Res 1979;3:363-70.
48. Delahaye NF, Rusakiewicz S, Martins I, Ménard C, Roux S, Lyonnet L, et al. Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. Nat Med 2011;17:700-7.
49. Walle T. Bioavailability of resveratrol. Ann N Y Acad Sci 2011;1215:9-15.
50. De Maria A, Bozzano F, Cantoni C, Moretta L. Revisiting human natural killer cell subset function revealed cytolytic CD56(dim)CD16+ NK cells as rapid producers of abundant IFN-gamma on activation. Proc Natl Acad Sci U S A 2011;108:728-32.
51. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood 2010;115:2167-76.
52. Lauwerys BR, Garot N, Renauld JC, Houssiau FA. Cytokine production and killer activity of NK/T-NK cells derived with IL-2, IL-15, or the combination of IL-12 and IL-18. J Immunol 2000;165:1847-53.
53. Brehm C, Huenecke S, Esser R, Kloess S, Quaiser A, Betz S, et al. Interleukin-2-stimulated natural killer cells are less susceptible to mycophenolate mofetil than non-activated NK cells: possible consequences for immunotherapy. Cancer Immunol Immunother 2014;63:821-33.
54. Ruiz-Medina BE, Ross JA, Kirken RA. Interleukin-2 Receptor β Thr-450 Phosphorylation Is a Positive Regulator for Receptor Complex Stability and

Activation of Signaling Molecules. J Biol Chem 2015;290:20972-83.

55. Gao Y, Moten A, Lin HK. Akt: a new activation mechanism. Cell Res 2014;24:785-6.
56. Julien LA, Carriere A, Moreau J, Roux PP. mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling. Mol Cell Biol 2010;30:908-21.
57. Whelan JT, Forbes SL, Bertrand FE. CBF-1 (RBP-J kappa) binds to the PTEN promoter and regulates PTEN gene expression. Cell Cycle 2007;6:80-4.
58. Lauder A, Castellanos A, Weston K. c-Myb transcription is activated by protein kinase B (PKB) following interleukin 2 stimulation of Tcells and is required for PKB-mediated protection from apoptosis. Mol Cell Biol 2001;21:5797-805.
59. Sireesh D, Dhamodharan U, Ezhilarasi K, Vijay V, Ramkumar KM. Association of NF-E2 Related Factor 2 (Nrf2) and inflammatory cytokines in recent onset Type 2 Diabetes Mellitus. Sci Rep 2018;8:5126.
60. Jia LF, Huang YP, Zheng YF, Lyu MY, Wei SB, Meng Z, et al. miR-29b suppresses proliferation, migration, and invasion of tongue squamous cell carcinoma through PTEN-AKT signaling pathway by targeting Sp1. Oral Oncol 2014;50:1062-71.
61. Chen CY, Jang JH, Li MH, Surh YJ. Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. Biochem Biophys Res Commun 2005;331:993-1000.
62. Shin HW, Lee YJ, Kim J. Role of c-Myb in the regulation of natural killer cell activity. Biochem Biophys Res Commun 2018;503:2807-13.
63. Varoni EM, Lo Faro AF, Sharifi-Rad J, Iriti M. Anticancer Molecular Mechanisms of Resveratrol. Front Nutr 2016;3:8.
64. Wiciński M, Socha M, Walczak M, Wódkiewicz E, Malinowski B, Rewerski S, et al. Beneficial Effects of Resveratrol Administration-Focus on Potential Biochemical Mechanisms in Cardiovascular Conditions. Nutrients 2018;10.
65. Kundu JK, Surh YJ. Cancer chemopreventive and therapeutic potential of

resveratrol: mechanistic perspectives. Cancer Lett 2008;269:243-61.

66. Lu CC, Lai HC, Hsieh SC, Chen JK. Resveratrol ameliorates Serratia marcescens-induced acute pneumonia in rats. J Leukoc Biol 2008;83:1028-37.

67. Wang XL, Li T, Li JH, Miao SY, Xiao XZ. The Effects of Resveratrol on Inflammation and Oxidative Stress in a Rat Model of Chronic Obstructive Pulmonary Disease. Molecules 2017;22.

68. Zhao X, Tong W, Song X, Jia R, Li L, Zou Y, et al. Antiviral Effect of Resveratrol in Piglets Infected with Virulent Pseudorabies Virus. Viruses 2018;10.

69. Cao G, Wang J, Zheng X, Wei H, Tian Z, Sun R. Tumor Therapeutics Work as Stress Inducers to Enhance Tumor Sensitivity to Natural Killer (NK) Cell Cytolysis by Up-regulating NKp30 Ligand B7-H6. J Biol Chem 2015;290:29964-73.

70. Spear P, Wu MR, Sentman ML, Sentman CL. NKG2D ligands as therapeutic targets. Cancer Immun 2013;13:8.

71. Fulda S, Debatin KM. Resveratrol-mediated sensitisation to TRAIL-induced apoptosis depends on death receptor and mitochondrial signalling. Eur J Cancer 2005;41:786-98.

72. Hu L, Cao D, Li Y, He Y, Guo K. Resveratrol sensitized leukemia stem cell-like KG-1a cells to cytokine-induced killer cells-mediated cytolysis through NKG2D ligands and TRAIL receptors. Cancer Biol Ther 2012;13:516-26.

73. Burns LJ, Weisdorf DJ, DeFor TE, Vesole DH, Repka TL, Blazar BR, et al. IL-2-based immunotherapy after autologous transplantation for lymphoma and breast cancer induces immune activation and cytokine release: a phase I/II trial. Bone Marrow Transplant 2003;32:177-86.

74. Cosco D, Paolino D, Maiuolo J, Marzio LD, Carafa M, Ventura CA, et al. Ultradeformable liposomes as multidrug carrier of resveratrol and 5-fluorouracil for their topical delivery. Int J Pharm 2015;489:1-10.

75. Ma L, Li W, Wang R, Nan Y, Wang Q, Liu W, et al. Resveratrol enhanced anticancer effects of cisplatin on non-small cell lung cancer cell lines by

inducing mitochondrial dysfunction and cell apoptosis. Int J Oncol 2015;47:1460-8.

76. Li Q, Huyan T, Ye LJ, Li J, Shi JL, Huang QS. Concentration-dependent biphasic effects of resveratrol on human natural killer cells in vitro. J Agric Food Chem 2014;62:10928-35.
77. Lee AM, Shandala T, Nguyen L, Muhlhausler BS, Chen KM, Howe PR, et al. Effects of resveratrol supplementation on bone growth in young rats and microarchitecture and remodeling in ageing rats. Nutrients 2014;6:5871-87.
78. Goldberg DM, Yan J, Soleas GJ. Absorption of three wine-related polyphenols in three different matrices by healthy subjects. Clin Biochem 2003;36:79-87.
79. Wenzel E, Somoza V. Metabolism and bioavailability of trans-resveratrol. Mol Nutr Food Res 2005;49:472-81.
80. Leischner C, Burkard M, Pfeiffer MM, Lauer UM, Busch C, Venturelli S. Nutritional immunology: function of natural killer cells and their modulation by resveratrol for cancer prevention and treatment. Nutr J 2016;15:47.
81. Davidson CJ, Guthrie EE, Lipsick JS. Duplication and maintenance of the Myb genes of vertebrate animals. Biol Open 2013;2:101-10.
82. Heikamp EB, Patel CH, Collins S, Waickman A, Oh MH, Sun IH, et al. The AGC kinase SGK1 regulates TH1 and TH2 differentiation downstream of the mTORC2 complex. Nat Immunol 2014;15:457-64.
83. Garofalo M, Condorelli GL, Croce CM, Condorelli G. MicroRNAs as regulators of death receptors signaling. Cell Death Differ 2010;17:200-8.
84. Ha TY. The Role of MicroRNAs in Regulatory T Cells and in the Immune Response. Immune Netw 2011;11:11-41.
85. Trotta R, Chen L, Ciarlariello D, Josyula S, Mao C, Costinean S, et al. miR-155 regulates IFN-γ production in natural killer cells. Blood 2012;119:3478-85.
86. Vargova K, Curik N, Burda P, Basova P, Kulvait V, Pospisil V, et al. MYB transcriptionally regulates the miR-155 host gene in chronic lymphocytic leukemia. Blood 2011;117:3816-25.
87. He Y, Jiang X, Chen J. The role of miR-150 in normal and malignant

hematopoiesis. Oncogene 2014;33:3887-93.

88. Chen Z, Stelekati E, Kurachi M, Yu S, Cai Z, Manne S, et al. miR-150 Regulates Memory CD8 T Cell Differentiation via c-Myb. Cell Rep 2017;20:2584-97.

89. Xiao C, Calado DP, Galler G, Thai TH, Patterson HC, Wang J, et al. MiR-150 Controls B Cell Differentiation by Targeting the Transcription Factor c-Myb. Cell 2016;165:1027.

90. Bi J, Tian Z. NK Cell Exhaustion. Front Immunol 2017;8:760.

91. Ndhlovu LC, Lopez-Vergès S, Barbour JD, Jones RB, Jha AR, Long BR, et al. Tim-3 marks human natural killer cell maturation and suppresses cell-mediated cytotoxicity. Blood 2012;119:3734-43.

ABSTRACT (IN KOREAN)

자연살해세포의 활성화에 의한 레스베라트롤의 항암효과 및 작용 기전 규명

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이유진

자연살해세포는 선천면역세포 중 하나로서, 바이러스 면역 및 항암 면역에 중요한 역할을 한다. 자연살해세포는 탁월한 악성세포 사멸능력을 가지고 있어 항암면역치료에 이를 이용하는 임상 및 비임상 연구들이 활발히 진행 중이다. 최근 임상연구에서는 자연살해세포의 활성화를 위해 저 용량의 Interleukin-2 (IL-2)와 면역증강 물질을 병용처리하는 요법도 시도되고 있다. 하지만 이러한 요법들은 독성을 나타내거나 IL-2의 효능을 상쇄시킨다는 제한이 있어 새로운 치료방법이

필요하다. 본 연구에서는 부작용을 최소화하면서 자연살해세포를 활성화 시킬 수 있는 물질을 찾고, 그 작용 기전을 규명하고자 하였다.

1장에서는, 부작용을 최소화하면서 자연살해세포를 활성화 시킬 수 있는 물질을 찾고자 저 용량의 IL-2와 자연 유래의 천연 화합물들을 병용처리하여 확인해 보았다. 여러 물질들 중 포도주에 많이 포함된 것으로 잘 알려진 레스베라트롤만이 특이적으로 자연살해세포의 주요 사이토카인인 IFN-γ의 생산 및 세포사멸능력을 증가시키는 것을 확인 할 수 있었다. 또한 자연살해세포의 활성화를 확인할 수 있는 지표인 CD107a와 암세포의 세포 사멸에 직접적인 연관을 가지고 있는 활성화 수용체인 NKp30, NKG2D 발현이 IL-2만 처리한 군에 비해 레스베라트롤을 병용 처리한 군에서 증가하는 것을 확인할 수 있었다. 세포주 실험에서뿐만 아니라 사람과 마우스 전혈에서도 레스베라트롤에 의해 자연살해세포의 활성이 증가하는 것을 확인하였다. 마지막으로 마우스 종양 모델을 이용해 레스베라트롤에 의해 활성화 된 자연살해세포로 인한 항암효과 및 항전이 효과 또한 확인 할 수 있었다. 이를 통해 레스베라트롤이 자연살해세포를 활성화시킴으로써 항암제로서의 효능을 기대할 수 있게 되었다.

2장에서는, 자연살해세포 활성화에 대한 레스베라트롤의 작용 기전을 규명하였다. 자연살해세포를 활성화 시키는 것으로 알려진 여러 사이토카인들과 병용 처리 해보았을 때, IFN-γ 분비에는 레스베라트롤이

모든 사이토카인들과 동반상승효과를 보였으나, 세포사멸능력에서는 IL-2만이 레스베라트롤과 동반상승효과를 보였다. 따라서 레스베라트롤이 IL-2 신호전달에 관여하는 인자들을 활성화 시킬 것이라 가정할 수 있었으며, 하위 인자들 중 Akt 신호전달에 관여하는 것을 확인 할 수 있었다. 더 나아가 레스베라트롤은 PTEN과 S6K1을 억제해 mTOR complex 2 (mTORC2) 복합체의 한 성분인 Rictor의 인산화를 억제함으로써 Akt 신호전달에 관여하는 것을 확인 할 수 있었다. 또한 레스베라트롤은 자연살해세포 활성 조절에 중요한 역할을 하며, Akt 및 mTORC2 신호전달의 하위 인자 중 하나인 c-Myb 전사 인자의 발현을 증가시키고 활성화 시키는 것을 알 수 있었다. 더 나아가 c-Myb 전사 인자의 기능을 억제하면 레스베라트롤의 효과가 없어지는 것을 확인함으로써 레스베라트롤이 c-Myb 전사 인자 의존적으로 작용하는 것을 확인하였다. 이러한 결과들을 종합해보면, 레스베라트롤은 저 용량의 IL-2와 병행 치료함으로써 기존의 고 용량 IL-2 투여 치료의 한계점을 극복할 수 있을 것으로 예상되며, 자연살해세포를 이용한 항암치료에 새로운 항암 보조제로서의 가능성이 기대된다.

핵심되는 말 : 자연살해세포, 레스베라트롤, IFN-gamma, Akt 신호전달, mTORC2 복합체, c-Myb 전사인자

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3. Shin HW, **Lee YJ**, Kim JS. Role of c-Myb in the regulation of natural killer cell activity. Biochem Biophys Res Commun 2018;503(4):2807-2813.