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Immunomodulatory activity of resveratrol: discrepant *in vitro* and *in vivo* immunological effects

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Abstract

trans-Resveratrol is a dietary polyphenolic compound present in grapes, which has been shown to exhibit strong anti-inflammatory, antioxidant, and chemopreventive activities. In this study we have compared the *in vitro* and *in vivo* effects of resveratrol on the development of various cell-mediated immune responses, including mitogen/antigen-induced T cell proliferation, induction of cytotoxic T lymphocytes (CTLs), interleukin-2 (IL-2) induced lymphokine activated killer cells, and cytokine production. We found significant suppression (>90%) of the mitogen/antigen-induced T cell proliferation and development of allo-antigen specific CTLs *in vitro* with resveratrol at a concentration of 25 μM. Intragastric administration of resveratrol (2 mg daily) to mice for 4 weeks showed no effect on age-related gain in body weight, peripheral blood cell counts (WBC, RBC, or platelets), or the cellularity of bone marrow or spleen. The $CD4^+$ and $CD8^+$ T cells in spleen or colony-forming units-total in the marrow also remained unaffected by treatment with resveratrol. Spleen cells, which were stimulated *in vitro* after being removed from mice which had been administered resveratrol for 2 or 4 weeks, showed no significant change in IL-2 or concanavalin A induced proliferation of T cells or production of IL-2 induced lymphokine activated killer cells. Further, the production of in interferon-gamma and IL-12 was not affected by administration of resveratrol, but production of tumor necrosis factor-alpha was reduced. Even when conducted entirely *in vivo*, treatment with resveratrol was found to only marginally reduce allo-antigen induced T cell proliferation and the generation of CTLs in the draining lymph nodes. Thus, even though resveratrol strongly inhibits T cell proliferation and production of cytolytic cells *in vitro*, oral administration of resveratrol for 4 weeks does not induce hematologic or hematopoietic toxicity, and only marginally reduces the T cell-mediated immune responses.

Keywords: Resveratrol; Immunomodulation; Cell-mediated cytotoxicity; Lymphocyte proliferation; Colony-forming units; Cytokines

1. Introduction

Plant-derived dietary constituents have been demonstrated to play an important role in the prevention of disease [1–5]. The beneficial effects of these dietary ingredients have been attributed, at least in part, to the presence of numerous polyphenolic compounds with antioxidant and free radical scavenging properties [6,7]. Epidemiological studies have shown a close association between a low incidence of coronary heart disease and breast cancer [8–10] and moderate consumption of red wine containing natural polyphenolic compounds. For this reason, resveratrol (3,4′,5-trihydroxystilbene), a non-flavonoid polyphenolic compound found in grapes, red wine, and peanuts has been studied for chemopreventive and antioxidant activity. Resveratrol has been shown to modulate lipoprotein metabolism [11,12], eicosanoid synthesis [13–15], lipid oxidation [16], and platelet aggregation [13,17]. Recently, resveratrol was demonstrated to inhibit cellular processes associated with tumor initiation, promotion, and progression [18]. It prevented the development of pre-neoplastic

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Abbreviations: CTLs, cytotoxic T lymphocytes; LAK cells, lymphokine activated killer cells; CFU, colony-forming units; IL-2, interleukin-2; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; ELISA, enzyme-linked immunosorbant assay; Con A, concanavalin A; LN cells, lymph node cells.

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lesions in mouse mammary glands in culture and reduced the incidence of carcinogen induced skin tumors in mice [18]. In other studies, resveratrol was shown to inhibit the proliferation of breast (estrogen-receptor positive and negative), oral, liver, and colon cancer cell lines *in vitro* in a dose- and time-dependent manner [19–22]. It also suppressed the induction of nitric oxide synthase and disrupted arachidonic acid metabolism by inhibiting cyclooxygenase-2 [23,24]. Resveratrol was shown to exhibit cardioprotective and neuroprotective activity in the rats [25,26].

We have previously reported that although resveratrol inhibits the growth of both normal hematopoietic progenitors and leukemic cells in a dose-related manner [27], however, unlike leukemia cells, the anti-proliferative effect of resveratrol on normal hematopoietic progenitor cells is less dramatic and reversible. Resveratrol induced program cell death or apoptosis in leukemia cells, but not in normal hematopoietic cells. In addition, hematopoietic progenitor cells treated with resveratrol *ex vivo* were fully capable of reconstituting hematopoiesis in lethally irradiated mice. Although the antiproliferative and anti-inflammatory activities of resveratrol have been well documented in many studies, little is known about its effects of on the production of cellular immune responses *in vitro* or *in vivo*. In an earlier study, we demonstrated that resveratrol down-regulates the development of T cell-mediated responses and production of cytokines *in vitro* [28]. The *in vivo* effects of resveratrol on the development of immune responses have not been adequately investigated. In the present study, we report hematological and hematopoietic effects of resveratrol administration to mice, as well as its effect on mitogen/antigen induced proliferation of splenic lymphoid cells, development of cytotoxicity, and the production of cytokines.

2. Materials and methods

2.1. Agents

trans-Resveratrol (*trans*-3,4′,5-trihydroxystilbene), mouse interleukin-2 (mIL-2) (2.5×10^8 U/mg), and Concanavalin A (Con A) were purchased from Sigma Chemical Co. For *in vitro* effects, a100 mM solution of resveratrol was prepared in DMSO and all test concentrations were prepared by diluting the appropriate amount of stock solution in tissue culture medium. For oral administration, resveratrol suspension in water was prepared at 10 mg/mL, and 0.2 mL of this preparation (2 mg) was administered to mice using a 20-G ballpoint oral feeding needle.

2.2. Mice

Eight- to 10-week-old male C3H (H-2^k) were purchased from Taconic Laboratories. Mice consumed Breeder Diet (W) 8626 (protein, 20.0%; fat, 10.0%; and fiber, 3.0%) and water *ad libitum* in the Bioresource Facility of the Henry Ford Health System. They were housed for at least 1 week before experimental use and age-matched animals were used within any given experiment. The treatment of mice and all experimental protocols were according to the Institutional Animal Care and Use Committee guidelines.

2.3. Tissue culture medium

All *in vitro* cell cultures were carried out in RPMI-1640 medium (Grand Island Biological Company), supplemented with 10% fetal calf serum (Hyclone), 1% penicillin/streptomycin, 25 mM HEPES buffer, and 5×10^{-5} M 2-mercaptoethanol. Hereafter, this medium will be referred as complete RPMI-1640 medium.

2.4. Preparation of spleen cells

Mice were killed by CO_2 inhalation and spleen was removed aseptically. Spleen was placed in cold Hank's balanced salt solution (HBSS) and teased apart with a pair of forceps and a needle. Single-cell suspension from the teased tissue was obtained by passing it through a 20-G needle. Cells were washed two times in cold HBSS and finally resuspended in complete RPMI-1640 medium. Cell viability was determined by trypan blue dye exclusion.

2.5. ^{3}H-Thymidine incorporation assay

To determine the effect of resveratrol on the proliferation of lymphocytes, 5×10^6 spleen cells were cultured in 5 mL of RPMI-1640 in a 25 cm^2 tissue culture flask in the absence or presence of mIL-2 (100 ng/mL) or Con A (1 μg/mL) or allogeneic spleen cells (1:1 ratio) as stimulators. Resveratrol was added to the cultures in concentration as described in the individual experiments. After incubation for 4 days at 37°, 95% humidity, and 5% CO_2, cells were washed once with cold PBS and resuspended in RPMI-1640 at 2×10^6 cells/mL. 0.1 mL of cell suspension was added to each well of a 96-well microtiter tissue culture plate in triplicate. 0.25 μCi of ^{3}H-thymidine in 20 μL HBSS was added to each well and the plate was incubated for additional 8 hr. Cultures were harvested with an automatic cell harvester using distilled water. The amount of radioactivity incorporated into DNA was determined in a liquid scintillation spectrometer.

2.6. Clonal growth assay for hematopoietic progenitor cells

For clonal growth of hematopoietic progenitor cells, 2×10^4 bone marrow cells in 1 mL of MethoCult 3534 (Stem Cell Technologies) were placed in 35 mm plastic petri dishes in duplicate. The MethoCult 3534 tissue culture medium consists of 1.1% methylcellulose in IMDM supplemented with mIL-3 (10 ng/mL), hIL-6 (10 ng/mL), and mSCF (50 ng/mL). This medium supports

the growth of colony-forming units (CFU)-GM, CFU-M, and CFU-G colonies. Dishes were incubated at 37° for 7 days and colonies developed (CFU-total), including CFU-GM (~60%), CFU-M (15–20%), and CFU-G (<10%) were scored under an inverted microscope.

2.7. In vitro generation of cytotoxic cells

2.7.1. Cytotoxic T lymphocytes (CTLs)

For the generation of allo-antigen specific CTLs, 10^7 spleen cells of C3H/HeN (responders) mice and an equal number of irradiated (20 Gy) allogeneic spleen cells of C57BL/6 (stimulators) mice were cultured in 10 mL of RPMI-1640 tissue culture medium supplemented with 10% FBS. After incubation for 5 days, cells were harvested and viability determined by trypan blue dye exclusion. Cells were tested for cytotoxicity against ^{51}Cr labeled EL-4 lymphoma cells of C57BL/6 origin as target cells in a 4 hr ^{51}Cr release assay as described below.

2.7.2. Lymphokine activated killer (LAK) cells

For the generation of non-specific cytotoxic LAK cells, C3H splenic cells (5×10^6 cells/mL) were cultured in medium alone or in the medium containing IL-2 (100 ng/mL). After incubation for 72 hr, cells were harvested and tested for cytotoxicity against 32Dp210 leukemia cells of C3H/HeN origin in a 4 hr ^{51}Cr release assay as described below.

2.7.3. Cytotoxicity assay

Target cells were resuspended at 1×10^7 cells/mL RPMI-1640 and 100 μCi Na^{51}Chromate was added to cells. Cells were incubated for 90 min at 37°. Following incubation, cells were washed three times in PBS to remove unbound radioactivity. The effector and labeled target cells were adjusted to desired cell concentrations and added to wells of a U-bottomed 96-well microtiter plate in triplicate to obtain effector:target (*E*:*T*) ratios of 100:1 to 12.5:1. For maximum release of radioactivity, target cells were lysed in 1% SDS solution. For minimal release of radioactivity (spontaneous release), target cells were incubated in medium alone. Plates were centrifuged at 800 rpm for 2 min and incubated at 37° for 4 hr. 100 μL of the supernatant from each well was removed to measure the amount of radioactivity released. Percent cytotoxicity was determined by the formula:

$$\text{Percent cytotoxicity} = \frac{\text{Exp. release (cpm)} - \text{Spon. release (cpm)}}{\text{Max. release (cpm)} - \text{Spon. release (cpm)}} \times 100$$

2.8. Production of cell supernatants for cytokine determination

2.8.1. Spleen cell supernatant

Spleen cells (2.5×10^6 cells/mL RPMI-1640) were treated with Con A (1 μg/mL) in the absence or presence of 50 μM resveratrol for 24 hr. In separate cultures, spleen cells were first treated with 50 μM resveratrol for 8 hr and then washed with PBS three times. Cells were then treated with Con A (1 μg/mL) for 20 hr and supernatant was collected by centrifugation. The concentration of mouse interferon-gamma (mIFN-γ) in cell supernatants was determined using commercially available cytokine-specific enzyme-linked immunosorbant assay (ELISA) kit (Bioresource International).

2.8.2. Macrophage supernatant

Thioglycolate induced peritoneal exudate cells (1×10^6) were plated in 60 mm petri dishes and allowed to adhere to the plastic surface for 1 hr. Non-adherent cells were removed and the adherent cells were treated with LPS (500 ng/mL) for 20 hr in the absence or presence of resveratrol (50 μM). In separate dishes, cells were first pretreated with resveratrol for 8 hr before stimulation with LPS. Culture supernatants were collected by centrifugation and analyzed for mouse tumor necrosis factor-alpha (mTNF-α) and IL-12 (p40) by ELISA.

2.9. Flow cytometric analysis

Cells were incubated with 5 μg/2×10^6 anti CD4-FITC or anti CD8-FITC (BD Biosciences) for 30 min at 4°. After washing in PBS containing 0.5% BSA, cells were analyzed by flow cytometry with a FACScan (Becton Dickinson).

2.10. Statistical methods

All values are expressed as means ± SD. One-way, two-way or three-way ANOVA, followed by *t* tests was the primary method used to test for differences between groups. Fisher's protected LSD method was used to address multiple comparisons. Differences were considered significant at $P < 0.05$.

3. Results

3.1. In vitro antiproliferative effect of resveratrol

The effect of resveratrol on proliferation of splenic lymphocytes was examined in ^{3}H-thymidine incorporation assay. The desired concentration of resveratrol was incorporated in culture medium at the initiation of cultures and was present during the entire period of incubation. The results presented in Fig. 1A and B demonstrate the effect of different concentrations of resveratrol (range, 6.25–50 μM) on IL-2 (A) and Con A (B) induced proliferation of splenic lymphocytes. There was no significant alteration of IL-2 induced proliferation of splenic cells at 6.25 or 12.5 μM resveratrol, but it was completely inhibited at 25 and 50 μM resveratrol ($P < 0.001$). Resveratrol also inhibited the Con A induced proliferation of splenic

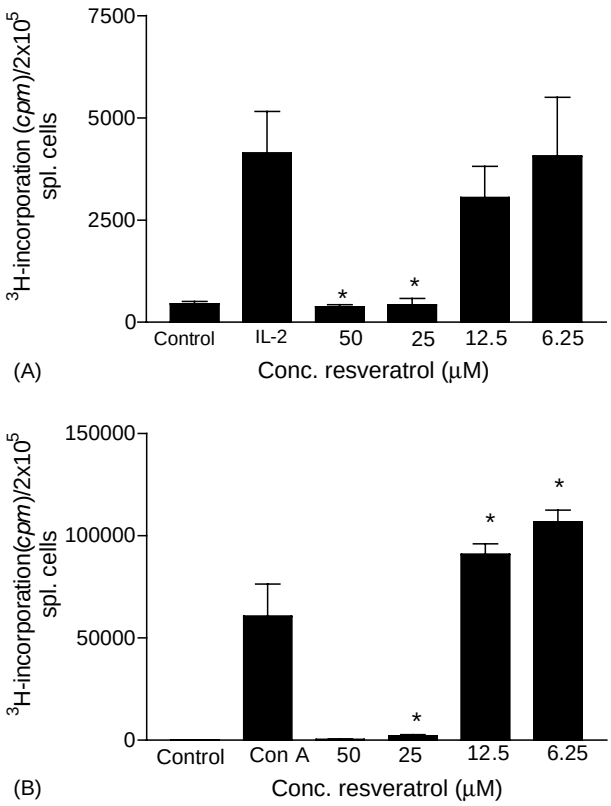

Fig. 1. Effect of resveratrol on proliferation of spleen cells. C3H/HeN spleen cells (2×10^5 cells/well) were stimulated with IL-2 (100 ng/mL) (A) or Con A (1 μg/mL) (B) in triplicate in 96-well microtiter tissue culture plates for 4 days in the absence or the presence of resveratrol (6.25–50 μM). Cultures were pulsed with ^{3}H-thymidine (0.25 μCi/well) for 8 hr and ^{3}H-thymidine incorporation was determined by liquid scintillation spectrometry. Data are presented as mean ± SD of three to four experiments.

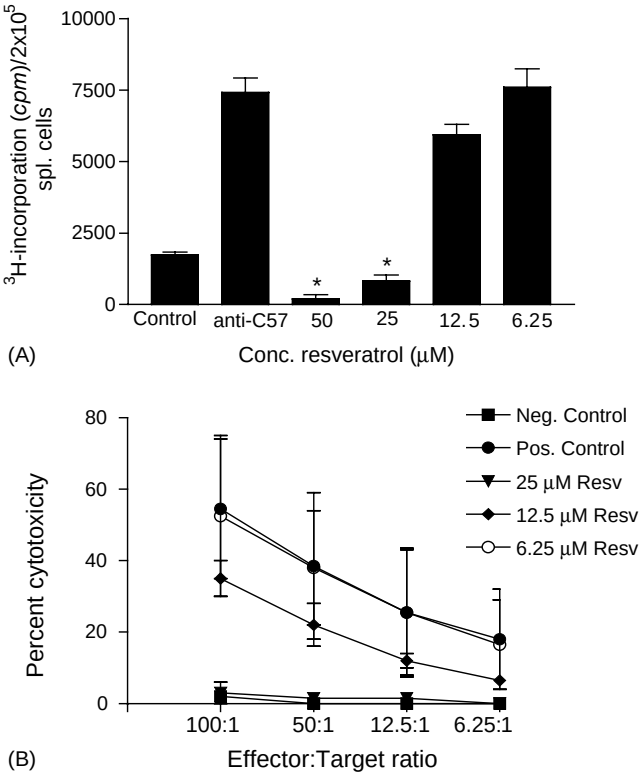

Fig. 2. Effect of resveratrol on the development of T cell-mediated immune responses. 1×10^7 C3H/HeN spleen cells were co-cultured with an equal number of irradiated C57BL/6 spleen cells in the absence or presence of resveratrol (6.25–50 μM). After incubation for 5 days, cells were harvested and tested for T cell proliferation (A) by ^{3}H-thymidine incorporation as described in Fig. 1. Cytotoxicity of the effector cells against EL-4 lymphoma cells of C57BL/6 origin was determined in a 4 hr ^{51}Cr-release assay at various effector:target (*E*:*T*) ratios (B). In each panel, the results are presented from a representative experiment. Similar results were obtained in three separate experiments.

cells (Fig. 1B). The effect of resveratrol on Con A induced proliferation of cells was dose-related, since complete suppression of proliferation was observed at 25 and 50 μM resveratrol ($P < 0.001$), however, proliferation of lymphocytes was significantly enhanced at 6.25 and 12.5 μM resveratrol ($P < 0.001$).

The effect of resveratrol on development of antigen specific cellular immune responses against allogeneic cells was investigated next. The effect on allo-antigen induced proliferation of spleen cells (C3H/HeN anti C57BL/6) was very similar to the effect on IL-2 induced proliferation, i.e. no effect at 6.25 μM, some inhibition at 12.5 μM, and complete inhibition of proliferation at 25 and 50 μM resveratrol ($P < 0.001$) (Fig. 2A). The effect of resveratrol on the development of allo-antigen specific CTLs correlated with its antiproliferative effect in MLR, i.e. complete inhibition of the CTL production at 25 μM, reduced suppression at 12.5 μM, and no effect at 6.25 μM resveratrol (Fig. 2B). These data demonstrate that resveratrol at concentration of 25 μM and above is highly suppressive of the Con A, IL-2, or allo-antigen induced proliferation of splenic lymphocytes, and the generation of allo-antigen specific CTLs.

The inhibitory effect of resveratrol on proliferative and cytotoxic responses was not due to DMSO used for dissolving resveratrol, since equivalent concentrations of DMSO alone had no effect on the proliferation of spleen cells or the development of cytotoxic responses (data not shown).

3.2. Hematologic and hematopietic effects of resveratrol administration

To investigate the effect of oral administration of resveratrol on clinically relevant parameters of toxicity, such as body weight, peripheral blood cell count, and cellularity of lympho-hematopoietic tissues, mice were administered resveratrol orally at 2 mg/day, 5 days/week, for 4 weeks. Body weight was recorded at various intervals and blood samples taken from the tail vein were analyzed with Coulter counter. All mice, up to 4 weeks of administration of resveratrol appeared normal without any evidence of ruffled fur or lethargy. There was no decrease in the body weight in treated mice compared to the body weight taken

Table 1
Hematologic effects of feeding resveratrol[a]

Group	WBC	RBC	Platelet	Hemoglobin
Control	15.7 ± 3.7	6.6 ± 0.7	1.2 ± 0.17	11.2 ± 1.5
2-week resveratrol	13.4 ± 3.0	6.4 ± 0.9	1.29 ± 0.12	11.4 ± 1.4
4-week resveratrol	16.9 ± 3.6	6.1 ± 1.1	1.19 ± 0.16	10.5 ± 1.7

[a] Resveratrol dosage; 2 mg/day orally, 5 days/week. WBC = 10^6 mL^{-1}; RBC = 10^6 μL^{-1}; platelets = 10^6 μL^{-1}; hemoglobin, g/dL.

prior to the administration of resveratrol, although the weight gain in the resveratrol group was slightly less than for the age-matched control mice (2.1 g vs. 2.9 g). Insignificant decrease (∼15%) in the WBC count was observed after 2 weeks of treatment, which was reversed by the end of 4 weeks of treatment (Table 1). There was no change in RBC or platelet counts at any time during the course of treatment with resveratrol. Similarly, hemoglobin also remained stable in treated mice (Table 1). To examine the effect of resveratrol administration on bone marrow and spleen, these organs were harvested 2 and 4 weeks after treatment with resveratrol and the number of nucleated cells determined with a hemocytometer. As shown in Table 2, there was no difference in the number of nucleated cells recovered from bone marrow (2 tibiae + 2 femurs) of mice treated with resveratrol for 2 or 4 weeks compared to control mice. There was an insignificant increase in nucleated cells (∼15%) in spleen cells after 2 weeks of treatment, but the count returned to the pretreatment level after 4 weeks. In addition, flow cytometric analysis showed no change in CD4 and CD8 subsets of T cells in spleen after 2 or 4 weeks of treatment with resveratrol.

3.3. Effect of resveratrol on hematopoietic progenitors

Whether resveratrol affects the hematopoietic progenitor cells was measured by determining the colony-forming units (CFUs) in the marrow of the treated mice. Bone marrow was harvested 2 or 4 weeks after administration of resveratrol and placed in tissue culture for the clonal growth of hematopoietic progenitor cells. Data demonstrate that treatment with resveratrol for 2 or 4 weeks does not alter the CFU-total contents in the marrow (Fig. 3), indicating that resveratrol does not decrease or increase the number of hematopoietic progenitor cells.

Table 2
Effect of feeding resveratrol on the cellularity of bone marrow, spleen, and splenic $CD4^+$ and $CD8^+$ T cell subsets[a]

Group	Bone marrow ($\times 10^6$)	Spleen ($\times 10^6$)	% splenic	
			$CD4^+$	$CD8^+$
Control	20 ± 2.1	73 ± 7	26.5 ± 4	10.1 ± 1.9
2 week resveratrol	19.3 ± 2	88 ± 17	25.1 ± 9.4	10.7 ± 3.8
4 week resveratrol	19.1 ± 2	71 ± 11	26.1 ± 4.8	9.2 ± 2.6

[a] Resveratrol dosage; 2 mg orally/day, 5 days/week.

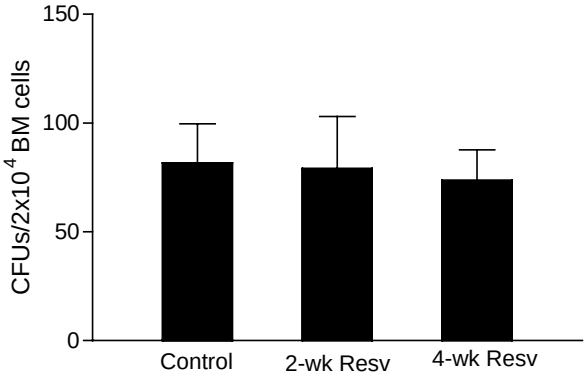

Fig. 3. Effect of resveratrol on colony-forming units-total (CFU-total) in bone marrow. Mice were administered resveratrol orally at 2 mg/day, 5 days/week for 4 weeks. Bone marrow cells (tibia and femur) from normal and treated mice were collected and tested for CFUs-total (CFU-GM, CFU-M, and CFU-G) by plating 2×10^4 BM cells in 1 mL MethoCult 3534 tissue culture medium in 35 mm petri plates in duplicate. Petri plates were incubated for 7 days and CFUs were scored under an inverted microscope. Identical results were obtained in three different experiments.

3.4. Effect of oral administration of resveratrol on the development of immune responses

Studies described above demonstrated that resveratrol inhibits the proliferative and cytotoxic immune responses of splenic lymphocytes *in vitro*, suggesting that administration of resveratrol may also suppress production of these responses *in vivo*. In order to test this, mice were treated with resveratrol orally for 2 or 4 weeks as described, and spleen cells taken 16–20 hr after the last treatment with resveratrol were examined for mitogen/allo-antigen induced proliferation in T cells, development of cytotoxicity and production of cytokines. There was no significant change in the IL-2 induced proliferative response in spleen cells of mice treated for 2 or 4 weeks (Fig. 4A) nor in the proliferative response induced with Con A (Fig. 4B). Similarly, the induction of IL-2 induced LAK cell-mediated cytotoxicity in spleen cells also remained unchanged after treating mice with resveratrol for 2 or 4 weeks (Fig. 5), indicating that oral administration of resveratrol had no effect on the capacity of spleen cells to generate LAK cell-mediated cytotoxicity.

3.5. Effect of feeding resveratrol on production of cytokines

Since cytokines play an important role in lymphocyte proliferation and development of cell-mediated cytotoxicity, the effect of treatment with resveratrol on production of cytokines was measured. For this purpose, mice were treated with resveratrol for 4 weeks and Con A induced production of IFN-γ from the spleen cells or LPS induced production of TNF-α and IL-12 from peritoneal macrophages was measured by ELISA. Data in Table 3 demonstrate that Con A induced production of IFN-γ by spleen cells of treated mice was not different from spleen cells of untreated control mice.

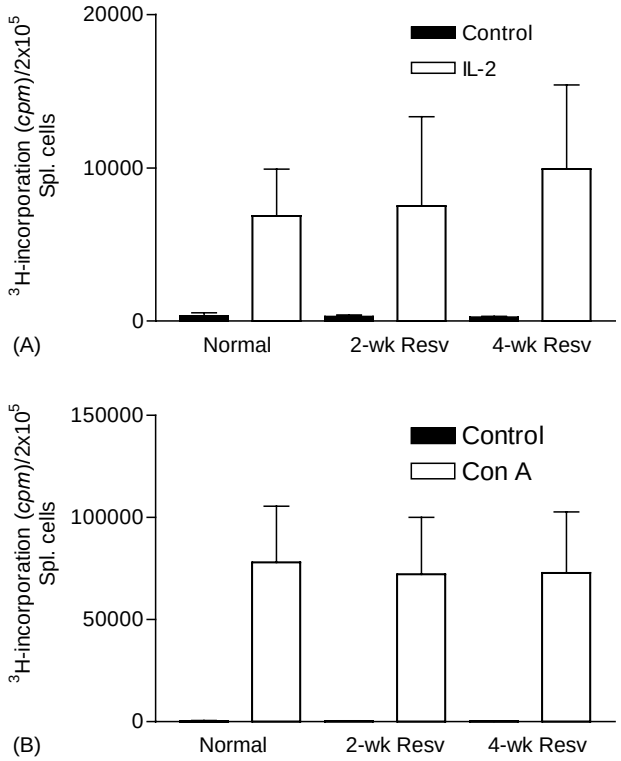

Fig. 4. Effect of resveratrol feeding on proliferative response in spleen cells. C3H/HeN mice were administered resveratrol orally at 2 mg/day, 5 days/week for 2 or 4 weeks. Spleen cells (2×10^5 cells/well) obtained from control and treated mice were stimulated with IL-2 (100 ng/mL) (A) or Con A (1 μg/mL) (B) in triplicate in 96-well microtiter tissue culture plates for 4 days. Cultures were pulsed with ^{3}H-thymidine (0.25 μCi/well) for 8 hr and ^{3}H-thymidine incorporation was determined by liquid scintillation spectrometry. Data are presented as mean ± SD of three experiments.

On the other hand, the LPS induced production of TNF-α ($P < 0.001$) but not IL-12 by peritoneal macrophages was reduced by treatment with resveratrol.

The results of the experiments described above demonstrated that unlike the strong suppressive effect of resveratrol

Table 3
Effect of resveratrol feeding on cytokine production[a]

Supernatant	Cytokine concentration (ng/mL)		
	IFN-γ	TNF-α	IL-12 (p70)
Nor-Spl cells[b]	0		
Nor-Spl cells/Con A	1685 ± 22	–	–
Resv-Spl cells	0	–	–
Resv-Spl cells/Con A	1678 ± 297	–	–
Nor-mι[c]	–	0	270.4 ± 18
Nor-mι/LPS	–	1642 ± 63	1064 ± 82
Resv-mι	–	0	212.9 ± 14
Resv-mι/LPS	–	1415 ± 36[*]	937.9 ± 58

[a] Resveratrol dosage; 2 mg orally/day, 5 days a week × 4 weeks.
[b] Spleen cells from normal or resveratrol-fed mice were cultured at 2.5×10^6 cells/mL for 24 hr with or without Con A (1 μg/mL).
[c] Peritoneal macrophages (1×10^6/2 mL per dish) from normal or resveratrol-fed mice were cultured for 24 hr with or without LPS (0.5 μg/mL).
[*] $P < 0.001$ vs. normal mι stimulated with LPS.

in vitro, feeding mice resveratrol for 2–4 weeks produced an insignificant immunosuppressive effect *in vivo*. Since lymphoid cells from treated mice were cultured *in vitro* to examine the induction of various immune responses, there is a possibility that cells recover from the suppressive effect of resveratrol while in culture. To more directly test the effect of resveratrol *in vivo*, the induction of allo-antigen induced proliferation and production of CTLs was carried out entirely in the animal. C3H/HeN mice that have been fed with resveratrol for 2 or 4 weeks were injected in the hind paws with 2.5×10^7 irradiated spleen cells from C57BL/6 mice. Five days later, the draining popliteal lymph nodes were removed and tested for proliferation by ^{3}H-thymidine incorporation and cytotoxicity against C57BL/6 target cells. The results demonstrate (Fig. 6A and B) that oral administration of resveratrol for 2 weeks insignificantly reduced the allo-antigen induced lymph node (LN) cell proliferation and development of CTLs (14 and 10%, respectively). The administration of resveratrol for

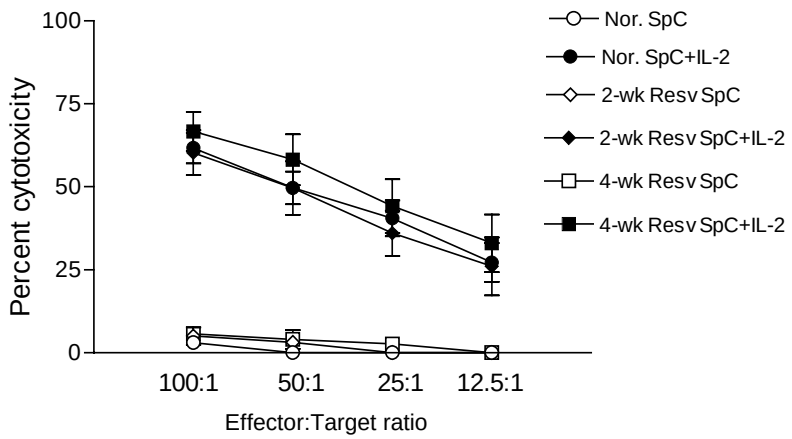

Fig. 5. Effect of feeding resveratrol on generation of cell-mediated cytotoxicity. C3H/HeN mice were administered resveratrol as described in Fig. 5. Spleen cells (5×10^6 mL^{-1}) from untreated control and resveratrol-fed mice were incubated with IL-2 (100 ng/mL) for 72 hr to generate lymphokine activated killer (LAK) cells. Cytotoxicity of LAK cells against YAC-1 target cells was measured in 4 hr ^{51}Cr-release assay at different *E*:*T* ratios. Similar results were obtained in two different experiments.

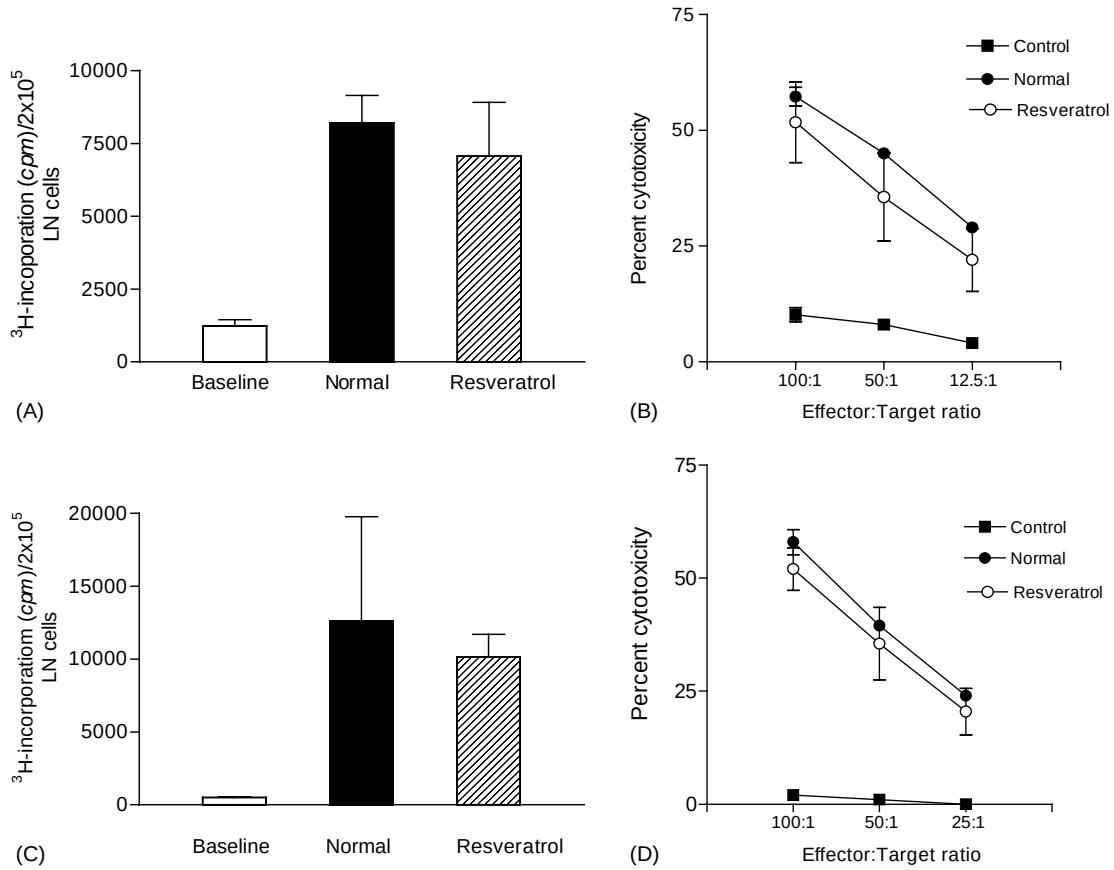

Fig. 6. Effect of resveratrol on *in vivo* development of T cell-mediated immune responses. Normal C3H/HeN mice or mice treated with resveratrol orally for 2 or 4 weeks as described in Fig. 5 were injected in the hind paws with 2.5×10^7 irradiated (20 Gy) C57BL/6 spleen cells. Five days later, draining popliteal lymph nodes were removed and tested for T cell proliferation by incorporation of ^{3}H-thymidine and cell-mediated cytotoxicity against EL-4 cells of C57BL/6 origin in 4 hr ^{51}Cr release assay. Top panel, T cell proliferation (A) and T cell-mediated cytotoxicity (B) after treatment with resveratrol for 2 weeks. Bottom panel, proliferative (C) and cytotoxic response (D) after treatment with resveratrol for 4 weeks. Similar results were obtained in two separate experiments.

4 weeks also only modestly (19 and 10%, respectively) reduced the development of these responses (Fig. 6C and D).

4. Discussion

Although resveratrol has been implicated in protection against chronic heart disease and chemoprevention of cancer [10,29], whether the mechanism(s) by which resveratrol provides protection against these diseases involves modulation of the immune system is not clear. In the present study, we investigated the effect of administering resveratrol orally on hemato-lymphatic tissues and the development of cell-mediated immune responses and production of cytokines. Administration of resveratrol at 8 mg/kg body weight results in plasma concentration, which is achieved by daily consumption of three glasses of red wine and is sufficient for cardioprotective effects [30]. In the present studies, resveratrol was administered at 80 mg/kg body weight, which is 10-fold higher than the cardioprotective dose, since in preliminary studies a dose of 8 mg/kg resveratrol was found to have no effect on the development of immune responses (data not shown). Our studies demonstrate that treatment with resveratrol at 80 mg/kg body weight for 2 or 4 weeks does not induce cachexia, hematologic toxicity or bone marrow suppression. There was no loss in body weight in the treated mice, and age-related gain in weight was comparable to control mice. Peripheral blood analysis revealed no major change in blood cell counts (WBC, RBC, or platelets) or hemoglobin after feeding mice resveratrol for 2 or 4 weeks. These results are in agreement with those previously reported by Juan *et al*. in which oral administration of resveratrol to rats for 28 days showed no effect on gain in body weight or peripheral blood cells [31]. The dose of resveratrol these investigators administered (20 mg/kg) was much smaller than 80 mg/kg in the present study, indicating that even at higher doses resveratrol does not adversely affect gain in body weight or blood cell counts.

Since lympho-hematopoietic tissues are the major sites where immune cells are produced, differentiate, and become functionally mature, the effect of resveratrol on cellularity of bone marrow and spleen was examined. There was no dramatic change in the cellularity of marrow or spleen whether mice were treated with resveratrol for 2 or 4 weeks. In addition, CD4 and CD8 subpopulations of

T cells in the spleen, both of which play a critical role in production of cell-mediated immune responses, were not affected following treatment with resveratrol. There was no evidence of hematopoietic toxicity by resveratrol either, since the number of CFU-total in bone marrow was not reduced after treatment with resveratrol for 2 or 4 weeks. Taken together, these data suggest that intragastric administration of resveratrol for 4 weeks does not produce hematologic or hematopoietic toxicity.

In vitro studies demonstrated that addition of resveratrol to cultures generally inhibits the proliferation of normal spleen cells induced with IL-2, Con A, or allo-antigens in a dose-related fashion. IL-2 or allo-antigen induced spleen cell proliferation was minimally affected at 6.25 or 12.5 μM resveratrol; however, at these concentrations of resveratrol the mitogen induced proliferative response was increased. The proliferative response of spleen cells to each of the three inducers was dramatically suppressed at 25 and 50 μM resveratrol. Resveratrol also exhibited a strong inhibitory effect on the development of allo-antigen specific CTLs *in vitro*. The inhibition of proliferation of spleen cells induced with mitogen/allo-antigen corroborates previously reported antiproliferative effect of resveratrol against several tumor cell lines [19–22]. Since resveratrol has been shown to inhibit ribonucleotide reductase and DNA polymerase activation, two key enzymes involved in DNA synthesis [32,33], as well as processes that are essential to allow cells to progress through the S phase of the cell cycle [34], the suppression of mitogen/antigen induced proliferation may also involve inhibition of these processes. In addition, the antiproliferative effect of resveratrol on lymphocytes may also be due to the antioxidant properties of resveratrol, since reactive oxygen species have been shown to have a role in cell response to cytokines and growth factors [35]. The suppression of allo-antigen specific CTL production by resveratrol is consistent with the results of a previous report in which resveratrol was shown to inhibit the generation of CTL and natural killer (NK) cell cytotoxic activity [36]. However, in contrast to our results, this report showed enhancement of the cytotoxicity by resveratrol at lower concentrations, indicating that resveratrol is capable of producing biphasic effects in a concentration-dependent manner.

To assess the effect of resveratrol on the development of cell-mediated immune responses *in vivo*, the splenic lymphoid cells of mice treated with resveratrol were investigated for the induction of several T cell-mediated immune responses, including mitogen/allo-antigen-induced T cell proliferation, cell-mediated cytotoxicity, and production of cytokines by T cells and macrophages. Investigation of the proliferative response of spleen cells of mice treated with resveratrol for 2 or 4 weeks following stimulation with IL-2 or Con A demonstrated that in contrast to the highly antiproliferative effect of resveratrol *in vitro*, *in vivo* administration of resveratrol to the mice does not significantly impair the ability of splenic T cells to respond to IL-2 or Con A. The proliferation of T cells induced by IL-2 was slightly reduced after 2 weeks of feeding resveratrol, but either returned to or slightly exceeded the response of control cells after administration of resveratrol for 4 weeks. Similarly, the production of IL-2 induced non-specific cytotoxic cells (LAK cells) was also not affected by administration of resveratrol for 2 or 4 weeks. Since cytokines play a prominent role in the development of immune responses, the effect of feeding resveratrol on production of IFN-γ by T cells or TNF-α and IL-12 (p40) by peritoneal macrophages was investigated. The results demonstrated that resveratrol whether administered for 2 or 4 weeks does not affect the production of IFN-γ by T cells or IL-12 (p40) by peritoneal macrophages, however, the production of TNF-α by macrophages was reduced significantly but not dramatically. The inability of resveratrol to compromise the production of these cytokines provides mechanistic explanation for the lack of strong suppressive effect of resveratrol on T cell-mediated immune responses *in vivo*. In contrast to the minimal effect of resveratrol on the development of immune responses *in vivo*, administration of resveratrol in the drinking water to Min mice for 7 weeks was shown to upregulate several genes involved in the recruitment and activation of immune cells, including CTL Ag-4, leukemia inhibitory factor receptor, and monocyte chemotactic protein 3 genes [37].

Because spleen cells from treated mice were cultured *in vitro* to study the effect of feeding resveratrol on the induction of various immune responses, it can be argued that cells recover from the *in vivo* suppressive effect of resveratrol when cultured *in vitro*. To rule out this possibility, the induction of allo-antigen stimulated T cell proliferation or CTLs in the draining lymph nodes of mice treated with resveratrol was investigated. The results of these experiments revealed 15–20% reduction in the proliferative response and ∼10% reduction in CTL response in the draining popliteal lymph nodes after feeding resveratrol for 2 or 4 weeks. Since intestinal absorption, plasma levels, metabolism, and disposition of resveratrol metabolites following oral administration have been well documented in rats, mice and humans by other investigators [38–41], it is unlikely that the lack of strong immunomodulatory effect of resveratrol in the present study is due to the lack of its bioavailability. The possibility remains that administration of resveratrol for a longer period of time may result in significant immunosuppression *in vivo*.

In conclusion, we have demonstrated that resveratrol effectively inhibits the mitogen/antigen induced lymphocyte proliferation and development of cell-mediated cytotoxicity *in vitro*, the development of cell-mediated immune responses or production of cytokines is only marginally reduced by administration of resveratrol *in vivo*. The results described here and those reported by others indicate that depending on the dose, route of administration, and the duration of treatment, resveratrol may exhibit effects ranging from no effect to suppression to upregulation of immune responses.

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