Resveratrol Prevents Endothelial Cells Injury in High-Do Therapy against Melanoma

Hongbing Guan, Narendra P. Singh, Udai P. Singh, Prakash S. Nagarkatti, Mitzi Nagarkatti

Abstract

Immunotherapy with high-dose interleukin-2 (HDIL-2) is an effective treatment for patients with metastatic melanoma a accompanied by severe toxicity involving endothelial cell injury and induction of vascular leak syndrome (VLS). In this s polyphenol with anti-inflammatory and anti-cancer properties, was able to prevent the endothelial cell injury and inhibit efficacy of HDIL-2 therapy in the killing of metastasized melanoma. Specifically, C57BL/6 mice were injected with B16 the next day and continued treatment with resveratrol once a day. On day 9, mice received HDIL-2. On day 12, mice We found that resveratrol significantly inhibited the development of VLS in lung and liver by protecting endothelial cell i undergoing apoptosis. The metastasis and growth of the tumor in lung were significantly inhibited by HDIL-2 and HDIL resveratrol co-treatment was more effective in inhibiting tumor metastasis and growth than HDIL-2 treatment alone. W $Gr\text{-}1^+CD11b^+$ myeloid-derived suppressor cells (MDSC) and $FoxP3^+CD4^+$ regulatory T cells (Treg). We found that res function of MDSC which inhibited the development of VLS after adoptive transfer. However, resveratrol suppressed th also found that resveratrol enhanced the susceptibility of melanoma to the cytotoxicity of IL-2-activated killer cells, and suppressor gene FoxO1. Our results suggested the potential use of resveratrol in HDIL-2 treatment against melanoma that MDSC is the dominant suppressor cell than regulatory T cell in the development of VLS.

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Introduction

High-dose interleukin-2 (HDIL-2) therapy which produces overall response rates of 15% to 23% remains an effective a cell carcinoma and melanoma [1], [2]. However, it is associated with significant systemic toxicity, mainly, vascular leak vascular permeability and decreased microcirculatory perfusion that causes extensive fluid retention in multiple organs failure [3], [4], [5] Although it is dose-dependent and reversible with therapy discontinuation, there is no specific and ef damage is the major feature of the vascular pathology. Direct effects of IL-2 on endothelial cells [6], [7], or through inc TNF-α, IL-1 and IFN-γ [3], have been previously reported. Otherwise, the cytotoxic effects by lymphokine-activated ki injury [8], [9], [10], [11].

Immunoregulation associated with the development of IL-2-induced VLS is not known. Our lab has reported that T reg IL-2 treatment; they suppress the cytolytic killing of endothelial cells by LAK cells in the *in vitro* experiments, thereby s regulation on the development of HDIL-2-induced VLS [12]. Lately, functional importance of myeloid-derived suppresse been appreciated. MDSC are a heterogeneous population of cells consisting of myeloid progenitor cells and immature co-expression of the myeloid lineage differentiation molecule Gr1 and CD11b. These cells markedly expand systemica various infectious diseases and some autoimmune diseases [13], [14]. Accumulating evidence demonstrates that their negative regulation of immune responses including adaptive and innate immunity, such as suppressing various T-cell fu [15], [16], [17], [18]. However, the role of MDSC in HDIL-2-induced inflammation and the development of VLS have no

Resveratrol, a naturally occurring polyphenol found in grapes and red wine, is widely used in animal models and posse effects including anti-infective, anti-inflammatory, and antioxidant properties. These interesting properties confer the ca protect endothelial function on resveratrol [19], [20]. In cancer patients, resveratrol exhibits anticancer properties, suc induction of tumor cell apoptosis, increase in chemosensitization of tumor cells, and exerts chemopreventive effects [2 suppresses tumor growth by inducing apoptosis in tumor cells through aryl hydrocarbon receptor (AhR) and by recipro [23]. For melanoma, experiments showed that resveratrol can enhance chemical cytotoxicity to the tumor, and suppre interruption and apoptosis [24], [25]. Nevertheless, effects of resveratrol on the development of VLS in HDIL-2 therap

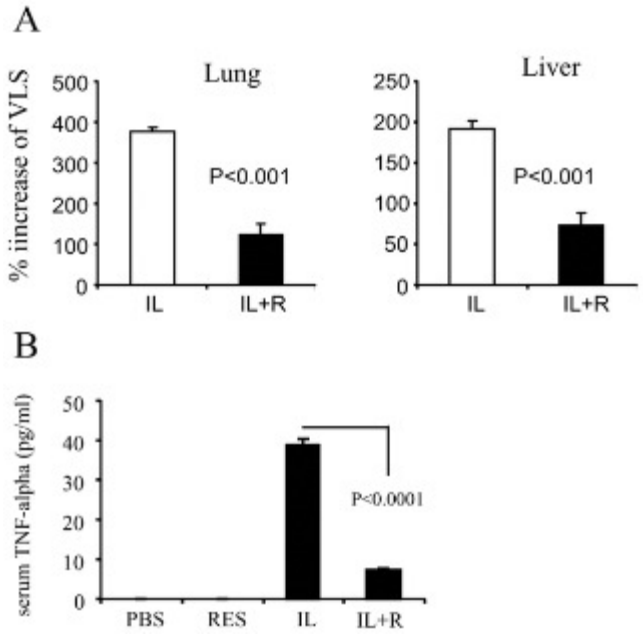

Figure 1. Decreased VLS in resveratrol-treated non-tumor mice.
Non-tumor bearing mice were given HDIL-2 and/or resveratrol. The vascular leak in the lungs and liver as well as T in lung. B. VLS in liver. C. The TNF-α level in the serum was measured by sandwich ELISA.
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In this report, we studied the role of resveratrol in the development of VLS in B16F10 melanoma-bearing mice and rev and suppression of metastasis. We also discovered a novel functional characteristic of resveratrol in induction of supp FoxO1 transcripts in context of suppressing the development of VLS and the growth and metastasis of B16F10 melan

Materials and Methods

Ethics statement, mice and recombinant IL-2

Female C57BL/6 (6–8 wk of age) mice were purchased from NIH. All animals were housed in University of South Car were performed according to NIH guidelines under protocols approved by the Institutional Animal Care and Use Comn Recombinant IL-2 was provided by the NCI Biological Resources Branch (Rockville, MD). All antibodies were purchas were purchased from MP biomedical. Resveratrol was purchased from Sigma or Supelco (Bellefonte, PA).

Induction and quantification of VLS

VLS was induced by IL-2 according to the established method from our lab [12], [26], [27], [28]. On day 0, groups of 75,000 units of IL-2 or PBS as a control, 3 times a day for 3 consecutive days. On day 4, the mice received one injec with 0.1 ml of 1% Evan's blue in PBS. After 2 h, the mice were exsanguinated under anesthesia, and the hearts were livers were blanched. The lungs and livers were harvested and placed in formamide at 37°C overnight. The Evan's blu the absorbance of the supernatants at 650 nm with a spectrophotometer. For administration of resveratrol, mice were weight) on day -1 by gavage, and thereafter once a day 1 h before the first IL-2 injection during day 0 to day 4. The V as percentage of increase in extravasation of Evan's blue when compared with that of the PBS-treated controls and w IL-2-treated mice)-OD650 in the organs of PBS-treated controls)]/(OD650 in the organs of PBS-treated controls) ×10 vascular leak, and data from 4–5 mice were expressed as mean ±SEM.

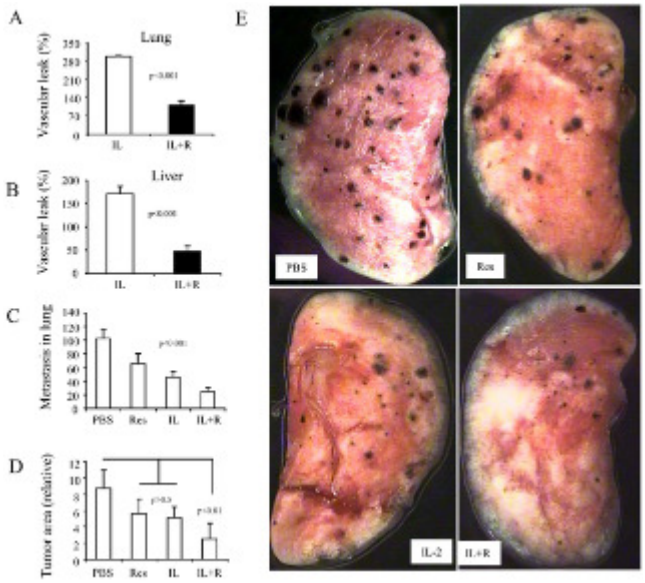

Figure 2. Decreased VLS and increased tumor regression in resveratrol-treated melanoma-bearing mice. C57BL/6 mice were injected *i.v.* with B16F10 melanoma cells (5×10^5 cells/mouse) and adminsitered resveratrol as treated with IL-2 as in Fig. 1. The mice were sacrificed for evaluation of VLS on day 12. The numbers of black me counted under a microdissecting microscope. The area of each nodule was measured with software Image Pro. T VLS in lung. B. VLS in liver. C. Enumeration of lung metastatic nodules. D. Area measurement of lung metastatic n metastasis on the surface of one lobe.
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Tumor cell implantation, HDIL-2 and resveratrol treatment

Mice were injected with 5×10^5 B16F10 cells intravenously or implanted with 2×10^5 B16F10 cells subcutaneously on da

purchased from American Type Culture Collection (Manassas, VA) and maintained as recommended by the supplier [2
on day 1 and induce VLS with HDIL-2 on day 9 as described above. On day 12, mice were sacrificed to evaluate VLS
were counted under a dissecting microscope on the surface of the lung specimens. These nodules exhibited character
melanomas (data not shown). The area of each nodule was measured with software Image Pro. The area of each no
same lobe and multiplied by 1000. The average area of the normalized measurement was present. For the primary tu
weighed, and then used for the total RNA extraction.

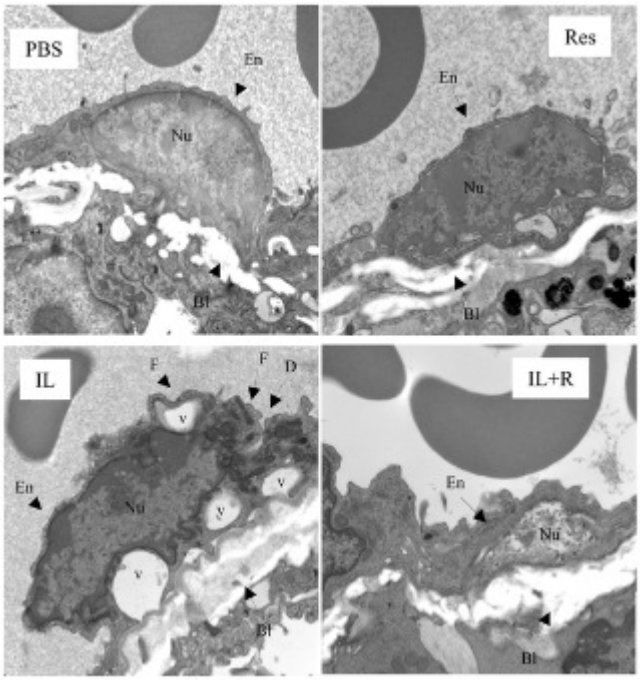

Figure 3. Resveratrol protects endothelial integrity.
The ultrastructural studies showed resveratrol protects endothelial cells. *Panels 1* and *2* show PBS- or resveratrol
cell (En) lining the blood vessel is firmly anchored to the basal lamina (BL) with good integrity, thereby showing nor
nucleus (Nu) and is closely opposed to the intact basal lamina. Three RBCs lie within the blood vessel lumen. *Pane*
damage to the endothelial cells. Cell membrane is folding (F) and discontinuous (D). Vacuolation (V) is found under
cytoplasm. In addition, some endothelial cells have lost the normal morphology, with only extended cell membrane
former endothelial cells can be found in the blood vessel lumen. An RBC fills the lumen. *Panel 4* shows IL-2+resve
cell morphology. The cell membrane and cytoplasmic contents are well defined and closely adhere to the basal lan
to the intact basal lamina Three RBC fill the lumen. Original magnification, ×20,000.
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Flow cytometry studies of MDSC and Treg

For flow cytometry analysis, splenocytes were prepared. Red blood cells were lysed using red blood cell lysing buffer
inflammatory cells were isolated using Percoll gradient centrifugation (detailed below). Cells were re-suspended in sta
2% fetal bovine serum) and pre-incubated for 10 min at room temperature with purified anti-CD16/32 antibody to bloc
receptors. For analysis of MDSC, cells were then incubated with FITC-conjugated anti-mouse CD11b and PE-conjuga
buffer for 30 min at 4°C. For analysis of Treg, cells were incubated with FITC-conjugated anti-mouse CD4 mAb in the
permeabilized with FoxP3 fix/perm buffer at room temperature in the dark for 20 min. The cells were washed once in
FoxP3 perm buffer at room temperature for 15 min. Cells were spun down and re-suspended in 100 µl fresh FoxP3 pe
PE-conjugated anti-mouse FoxP3 mAb at room temperature in the dark for 30 min. After incubation with the mAbs, ce
and re-suspended in the staining buffer. The staining was analyzed with a CXP500 flow cytometer with post-acquisitio
(Beckman Coulter). All antibodies were purchased from Biolegend.

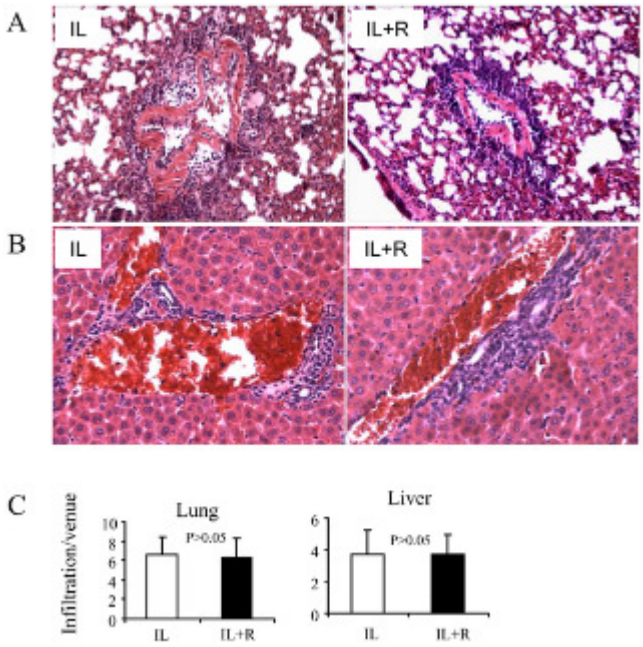

Figure 4. Histological studies of the lung and the liver for inflammatory cell infiltration.
Lungs and livers from VLS mice were harvested and preserved in 10% formalin solution. Sections were stained wi perivascular infiltration was determined by counting the number of cells infiltrating around a venule. The data depict individual mice. A. The level of perivascular infiltration in lungs. B. The level of perivascular infiltration in livers. C. S p>0.05 between IL-2 and IL-2+Resveratrol groups. Original magnification, ×200.
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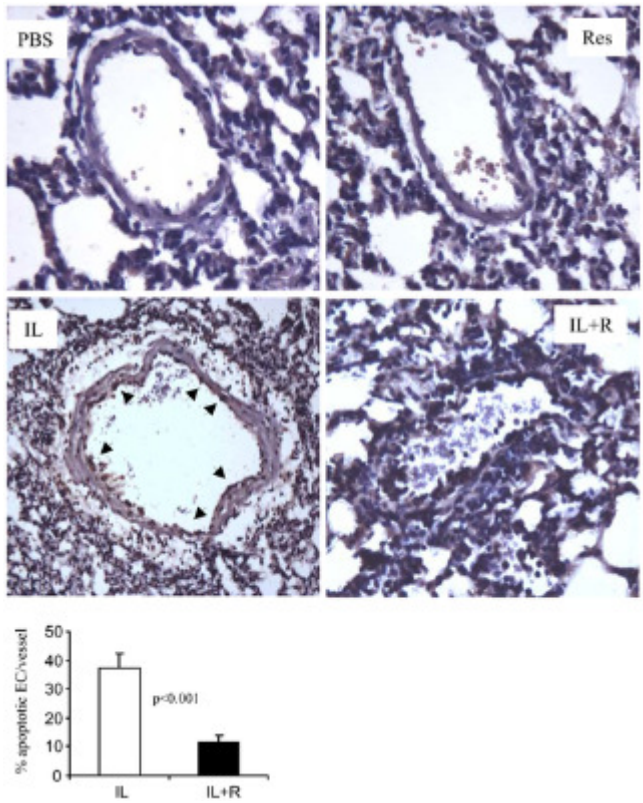

Figure 5. Resveratrol protects endothelial cells from apoptosis.
Lungs from PBS, resveratrol, IL-2 or IL-2+resveratrol-treated mice were examined for apoptosis with TUNEL assa
Apoptotic cells are depicted by brown stained nuclei (arrow). The histogram shows the quantification of apoptotic
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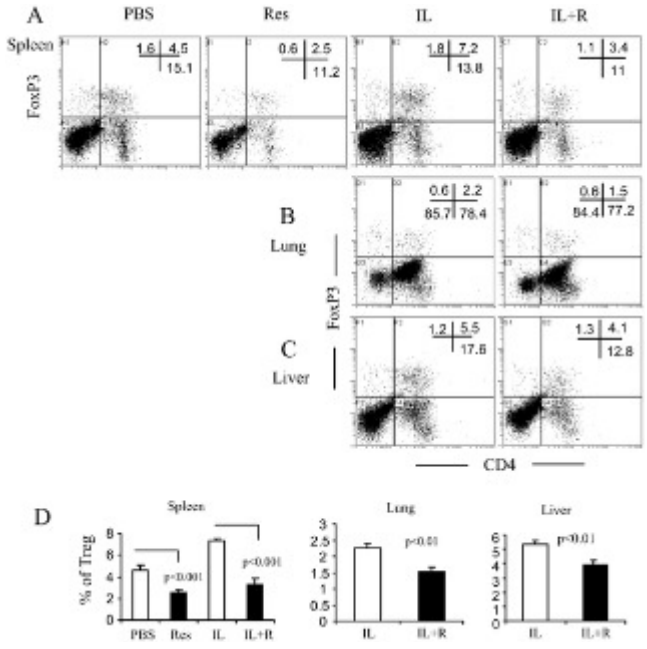

Figure 6. Resveratrol inhibits IL-2-induced expansion of Treg.
Splenocytes and infiltrating cells were stained with FITC-conjugated anti-CD4 mAb, fixed and permeabilized and in
mAb as described in Materials and Methods. The stained cells were analyzed by flow cytometry. The representati
liver (C) and the statistical analysis of the percentage mean ±SEM of Treg (D) are shown.
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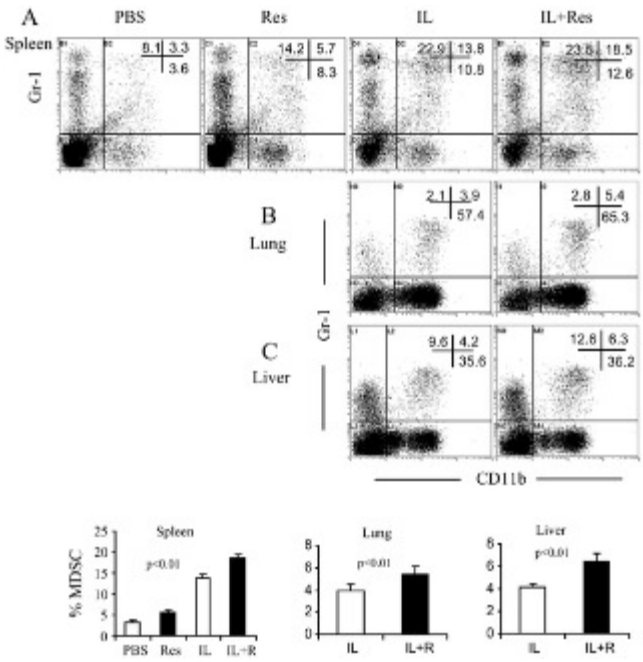

Figure 7. Expansion of MDSC by IL-2 and/or resveratrol treatment.
Splenocytes and the infiltrating cells were stained with FITC-conjugated anti-CD11b and PE-conjugated anti-Gr-1 r flow cytometry. The representative dot plots from spleen (A), lung (B), and liver (C) and the statistical analysis of t were shown.
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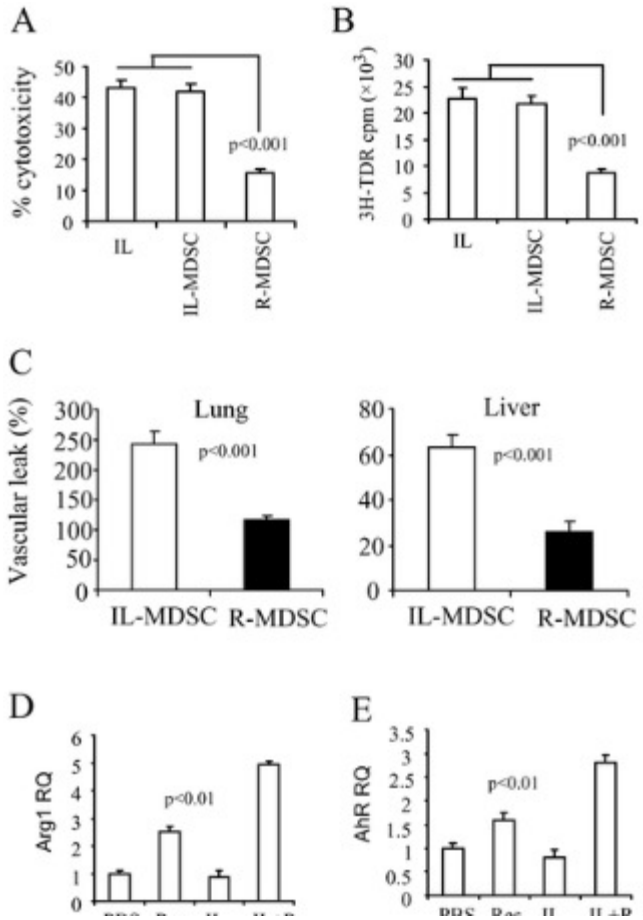

Figure 8. Analysis of the functional characteristics of MDSC.
Panel A: MDSC were isolated from IL-2 and IL-2+resveratrol treatment (labeled as IL-MDSC and R-MDSC respe were isolated and incubated with MDSC for 2 h, and then used as effectors in the cytotoxicity assay against EC ce incorporation assay to measure the proliferation of the LAK cells in the panel A. *Panel C:* 1×10^6 MDSC were intrav was then induced in the recipient mice. *Panel D & E:* Total RNA was extracted from the MDSC with the respective (E) were detected by QPCR. The relative expression was normalized to the endogenous 18S.
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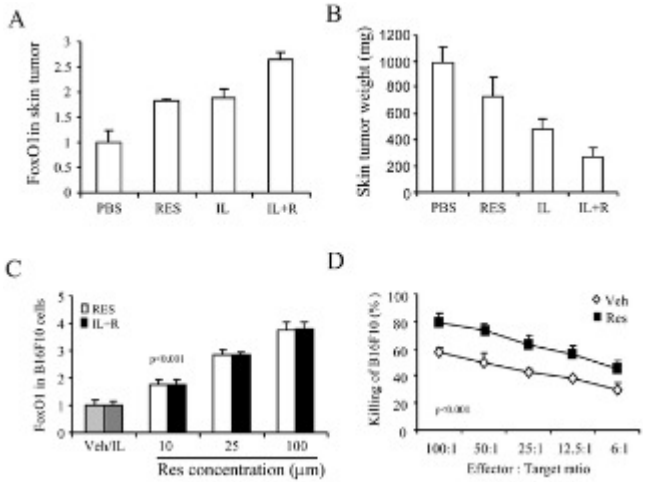

Figure 9. Resveratrol enhances the expression of FoxO1 and the cytolytic susceptibility of melanoma.
Panel A and B: C57BL/6 mice were implanted *s.c.* with B16F10 melanoma cells (2×10^5 cells/mouse) and orally ac were treated with IL-2 as described in Fig. 1. The mice were sacrificed for evaluation of VLS on day 12. The prima weighed. Total RNA was extracted. Expression of FoxO1 was detected by QPCR (A). The relative expression was mean ±SEM of weight is shown (B). *Panel C:* B16F10 cells were cultured with vehicle (Veh), IL-2 (IL) (1000 units/ 24 h. Total RNA was extracted. Expression of FoxO1 was detected by QPCR. *Panel D:* LAK cells were generated with IL-2 (1000 units/ml) for 48 h. B16F10 cells were incubated with resveratrol (25 μM) for 2 h and labeled with ^{5} added to the LAK cells. After 4 h, the release of ^{51}Cr was measured and percent cytotoxicity was calculated. Stat effector to target ratio and showed $p<0.001$.
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MDSC sorting and adoptive transfer

MDSC were sorted by two steps. First, MDSC cells were enriched from splenocytes of VLS mice by depletion of othe NK1.1+, F4/80+, CD11C+, CD117+) with magnetic beads, and then, $CD11b^{+}Gr\text{-}1^{+}$ double positive cells were sorted b density of 1×10^8 cells/ml in the staining buffer were pre-incubated for 10 min with purified anti-CD16/32 antibody to bl receptors, and then incubated with biotin-conjugated anti-mouse CD4, CD8, CD90.2, B220, NK1.1, F4/80, CD11c, an temperature. Cells were washed once with and re-suspended in the staining buffer at the same cell density. Cells wer (Stemcell) at room temperature for 15 min followed by incubation with EasySep magnetic particles (Stemcell) at room

cells were collected, and further incubated with FITC-conjugated anti-mouse CD11b and PE-conjugated anti-mouse G washed twice with and re-suspended in the staining buffer. CD11b and Gr-1 double positive cells were then sorted by adoptive transfer, 1×10^6 cells were transferred per recipient mouse intravenously.

Generation of LAK cells and ^{51}Cr release assay

LAK cells were prepared as previously described [12], [26], [27], [28]. For generation of LAK cells *in vitro*, splenocyte 1000 units/ml IL-2 in RPMI containing 10% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutar units/ml penicillin, 100 µg streptomycin, and 50 µM β-mercaptoethanol. Viable cells were purified by density gradient c Burlington, NC). Such cells will be referred to as LAK cells. For generation of LAK cells *in vivo*, mice were treated wit splenocytes were prepared and viable cells were purified using NycoPrep and referred to as LAK cells. Cytotoxicity o or TME endothelial cells using 4 h ^{51}Cr-release assay. Briefly, 1×10^6 target cells were labeled with 100 µCi of ^{51}NaCr three times in RPMI 1640 culture medium, and adjusted to 1×10^5 cells/ml. In brief, 1×10^4 target cells were plated into of LAK cells depending on the respective E:T cell ratios. In these experiments, the LAK cells were defined as the effe $^{51}NaCrO_4$-labeled endothelial cells or tumor cells, which were defined as the target cells (T). In some groups, target c for 2 h at 37°C before they were added into the plates. For analysis of MDSC suppression, LAK cells were incubated plates. Spontaneous release of $^{51}NaCrO_4$ was determined by culturing the target cells alone, and the maximum releas the target cells with 1% SDS. The supernatants were harvested after 4 h culture, and the radioactivity was measured Percentage of killing efficiency of LAK cells was calculated as percent cytotoxicity = [(sample cpm – spontaneous rele spontaneous release cpm)] ×100. The data were represented as mean ±SEM. Each group had four wells.

Isolation of T cells and infiltrating inflammatory cells

To isolate T cells from spleen, splenocytes were stained with anti-CD3 Abs, followed by magnetic beads isolation. Fo (MNCs) from lung or liver, mice were perfused. Lung or liver was minced and passed through 100 µm filter. Single-cel (70%/30%) centrifugation.

TUNEL assay of cell apoptosis

Cell apoptosis was evaluated with conventional TUNEL assay using DeadEnd Colorimetric TUNEL System from Prom instructions. Numbers of TUNEL-positive stained endothelial cells were counted in 5 vessels of each slide and the data cells per vessel.

Histological Analysis of cell infiltration and measurement of serum TNF-α

The lungs and livers were fixed in 10% formalin solution. The organs were embedded in paraffin, sectioned, and staine infiltration was scaled by counting the number of lymphocytes infiltrating around the vessel and averaging the minimum samples per mouse were analyzed, and a minimum of four mice were included. Sera were prepared for detection of T kit (Biolegend).

Electron microscopic studies of vascular injury

Tissue samples were fixed in 5% glutaldehyde/4.4% formaldehyde/2.75% picric acid in 0.05 M sodium cacodylate buf buffer, postfixed in osmium tetroxide, embedded in Polybed 812 resin (Polysciences, Warrington, PA) and studied with

[^{3}H]-thymidine incorporation assay

LAK cells generated from IL-2 treated mice were cultured in complete RPMI 1640 medium in a 96-well round bottom the culture. In some groups, LAK cells were incubated with MDSC at 10:1 ratio (LAK:MDSC) for 2 h before adding [^{3}H using a cell harvester and the radioactivity incorporated in the cells was measured in a beta counter (Microbeta counte

Quantitative real-time PCR (QPCR)

Total RNAs were prepared and reversely transcribed to cDNA using miRNeasy Mini Kit and miScript Reverse Transcri aryl hydrocarbon receptor (AhR), and 18S RNA (18S) were amplified using miScript SYBR Green PCR kit (Qiagen) ir relative gene expression level was normalized to the endogenous control 18S. The primer sequences were listed belo AGT TTA; FoxO1 R: GGT GAA AGC AAA GGG CTC CAA TGT; Arg1 F: ACG GCA GTG GCT TTA ATT GGC; Arg1 NOS2 F: TGC CAC CAA CAA TGG CAA CAT C; AhR R: TTG AAG TCA ACC TCA CCA GCA GC; 18S F: AAA GGA TAA GAA CGG CCA TGC ACC AC.

Statistical analysis

Data were presented as mean ±SEM and analyzed for significance using non-paired Student's t-test. In case of multip Newman-Keuls Multiple Comparision Test was applied using Graphpad Prism 5 software. Comparisons were conside

Results

Resveratrol prevents VLS while maintaining the effectiveness of HDIL-2 treatment

HDIL-2 has been shown to have significant efficacy against advanced stages of melanoma. However, HDIL-2 treatme including VLS which limits its therapeutic efficacy [1], [2], [3], [4], [5]. In the current study, we first evaluated whether r HDIL-2-induced VLS. For this purpose, we induced VLS in normal mice with HDIL-2. Resveratrol was given to mice or day during the treatment until the day of sacrifice of mice. Four sets of experiments were established: IL-2 treatment resveratrol co-treatment (IL+R), and PBS treatment (PBS). The results showed that the vascular leak was dramatical levels of TNF-α were also significantly decreased in resveratrol treatment group (Fig. 1). These results demonstrated

Next, we tested whether resveratrol exerts a similar effect in melanoma-bearing mice. To this end, C57BL/6 mice wer (5×10^5 cells/mouse). On day 9, mice were treated with IL-2 or resveratrol as described above. The mice were euthar results showed that resveratrol significantly inhibited VLS in tumor-bearing mice both in the lungs and liver to a similar non-tumor-bearing mice (Fig. 2A and 2B). The metastasis and tumor growth in lungs (seen as black nodules) were sig co-treatment. Moreover, IL2+resveratrol co-treatment was more effective in inhibiting tumor metastasis than IL-2 trea indicated that resveratrol can ameliorate IL-2 toxicity while promoting the effectiveness of IL-2 in metastatic melanoma

Resveratrol does not affect infiltration of inflammatory cells but protects endothelial ce from undergoing apoptosis

We previously found that HDIL-2-therapy caused endothelial cell (EC) damage by activating LAK cells [26], [28], [29]. mediated suppression of VLS resulted from its protective effect on ECs by pursuing ultrastructural studies of the lungs resveratrol group showed normal morphological features. In contrast, ECs from IL-2-treatment group revealed extens break and vacuolation of cytoplasmic particles. Some of the endothelial cells were severely damaged, with only exten Cellular debris from ECs was found in the blood capillary lumen [26]. Interestingly, IL2+resveratrol treatment exhibited lamina, whereas we only found minor damage in few endothelial cells.

We wondered whether suppression of IL-2 induced VLS by resveratrol resulted from inability of inflammatory cells to i inflammatory cell extravasation was examined under microscope. The results showed that the IL-2 treatment induced and liver. Notably, IL-2 + resveratrol co-treatment also exhibited similar levels of perivascular infiltration as the IL-2 tre measured by counting the layers of cell infiltration around each vessel and averaging the numbers for each group (Fig. suppression of VLS seen in IL-2 + resveratrol treated-mice was not due to the inability of infiltration of inflammatory ce

We previously reported that apoptosis could be one of the mechanisms of EC damage [26]. Hence, we applied TUNE Our results showed that IL-2-treated mice exhibited a large number of ECs that had undergone apoptosis, which was

contrast, IL-2+resveratrol treated-mice did not show TUNEL-positive cells (Fig. 5). These data suggested that resvera ultrastructural damage and apoptosis.

Resveratrol induced expansion and suppressive functionality of myeloid-derived supp

To explore the underneath mechanisms that contribute to the suppressive function of resveratrol on VLS, we studied tl inhibition of VLS by resveratrol. To this end, we focused on studies of Treg and MDSC. Our previous study showed th $CD4^+FoxP3^+$ Treg; these Treg can suppress the cytolytic killing of ECs by LAK cells *in vitro* [12]. In the current study, spleen, lung and liver. On day 12, the splenocytes and the infiltrating-mononuclear cells from lung and liver were prepa showed that IL-2 caused an expansion of Treg in spleen, lung and liver. However, resveratrol treatment did not induce expansion to some extent (Fig. 6). Simultaneously, we stained cells for Gr-1 and CD11b expression. The results show the expansion of MDSC. Moreover, combination of IL-2+resveratrol caused a further induction of MDSC (Fig. 7). Thes rather than Treg probably contributed to the suppression of VLS in resveratrol treatment.

To address this further, we examined the suppressive ability of MDSC on LAK cytotoxicity and proliferation. To this en isolated, and then incubated with MDSC at 10:1 ratio (LAK: MDSC) for 2 h at 37°C. For the cytotoxicity assay, the mi EC target (TME cell line) at 50:1 ratio (E:T) followed by 4 h ^{51}Cr-release assay. For the proliferation assay, the mixtur ^3H-thymidine and continued culture for 16 h and then the ^3H-thymidine incorporation was measured. To our surprise, th IL-2+resveratrol group (named as R-MDSC) significantly inhibited LAK cytotoxicity and proliferation; however, MDSC show any suppressive effects on either LAK cytotoxicity or proliferation (Fig. 8A & 8B).

To further evaluate whether these MDSCs play a suppressive role in the development of VLS *in vivo*, we transferred t induced VLS in the recipient mice on the same day of the cell transfer. The results showed that R-MDSC caused mark inhibit VLS at all (Fig. 8C). Correspondently, we found that resveratrol induced the expression of Arg1 in MDSC but IL co-treatment induced the most expression of Arg1 (Fig. 8D). This could be because resveratrol is more effective in ac also found that R-MDSC and IL-MDSC have different expression level of AhR, which is the main receptor for resverat resveratrol increased the expression of AhR in MDSC but IL-2 did not. Similar to the expression of Arg 1, IL-2 and res expression of AhR (Fig. 8E). All together, our results indicated that resveratrol can elicit or enhance the suppressive fu the suppression of VLS.

Resveratrol induced expression of FoxO1 in tumor cells

Thus far, we demonstrated that resveratrol can ameliorate IL-2 toxicity while promoting the effectiveness of IL-2 in me resveratrol-treated MDSC could contribute to the suppression of the cytolytic activity of LAK cells on ECs. However, M helping evasion of tumor by suppressing the host immunity in situ of the tumor. Our results raised the possibility that re that can overcome the suppressive effect of MDSC to the tumor. To explore the possible mechanisms, we studied wh expression of the tumor suppressor genes by analysis of FoxO1 gene expression. To this end, we resected the prima transcripts of FoxO1 by QPCR. The results showed that either resveratrol or IL-2 treatment can induce the expressio resveratrol co-treatment induced the highest expression of FoxO1 (Fig. 9A). Correspondently, the growth of the tumor which was demonstrated by the decreased tumor weight while IL-2 and resveratrol co-treatment can exert the maxim 9B).

We also cultured B16F10 cells with IL-2 and/or different dose of resveratrol. We found that resveratrol could induce th manner. However, IL-2 alone did not induce the expression of FoxO1. This concentration of IL-2 is effective in LAK cel co-treatment, the expression levels of FoxO1 were not different from the resveratrol treatment alone (Fig. 9C), which expression of FoxO1 in B16F10 tumor cells. However, in the *in vivo* experiment, HDIL-2 administration did induce the (Fig. 9A). These results indicate that the release of other factors from the HDIL-2-induced inflammation could trigger t factors work together with resveratrol co-treatment to further enhance the expression of FoxO1.

Resveratrol makes melanoma more susceptible to the cytotoxicity of LAK cells by inc cells to the cytotoxicity

It has been shown that resveratrol can suppress melanoma growth by inducing the cell-cycle interruption and apoptos

enhance the chemical cytotoxicity to the tumor by the chemotherapeutic agents [24], [25]. In this study, we studied wh cytotoxicity to the tumor. To this end, LAK cells were used as the effectors and generated *in vitro* from splenocytes of *Methods*. B16F10 cells were used as the targets. We pre-incubated the targets with resveratrol at 25 μM for 2 h at 3 results showed that the pre-treatment of resveratrol significantly increased the number of the killed targets by LAK ce

The above results suggested that resveratrol can directly induce the expression of the tumor suppressor gene FoxO1, tumor growth and cell death by triggering autophagy [31], [32]. IL-2 can facilitate such effects by the unknown factors Meanwhile, resveratrol directly enhances the susceptibility of B16F10 tumor cells to the LAK cytotoxicity. Together, the suppressive activity of MDSC against the immunity of the host that eventually leads to the tumor regression.

Discussion

Our results support a potential use of resveratrol in HDIL-2 treatment against melanoma and revealed some mechanis inhibited the development of VLS that is the most severe side-effect from HDIL-2 therapy. Meanwhile, the co-treatmer efficacy of the tumor therapy arisen from either resveratrol or HDIL-2 treatment. Resveratrol played a differential role tumor. On the one hand, resveratrol prevented ECs from the ultrastructural damage and apoptosis; on the other hand, susceptibility of the tumor to the cytotoxicity killer cells.

To understand how resveratrol played the suppressive role in the development of VLS, we focused on the study of Tre immune suppressors. We found that resveratrol induced the expansion of MDSCs that can suppress the cytolytic killin number of Treg decreased at the same time. Previous studies from our lab and the others also showed that resveratr expansion of Treg in the tumor-bearing mice while it induced the expansion of MDSCs that suppressed the developme demonstrate that MDSC is the predominant suppressor cells than Treg in the development of VLS. It could be true sir that induce the tumor escape than Treg [35]. This conclusion could be corroborated by the fact that HDIL-2 treatment not able to prevent the development of VLS [12].

There are conflicting reports on whether MDSC are involved in the induction of Treg [13]. It was also reported that bo bearing mice, and that the expansion of the two populations were not related [36]. Here, we showed that IL-2 treatme However, resveratrol treatment only induced MDSC expansion while it inhibited Treg expansion. Therefore, our current resveratrol on Treg and MDSC.

The expansion of MDSC in HDIL-2 immunotherapy was found in patients with renal cell carcinoma (RCC) therapy. In t in the patients blood was also detected [37], [38]. However, the role of MDSC in the development of HDIL-2-induced V before. In the current report, we found that HDIL-2-induced MDSCs from spleen were not suppressive. This is not sur naïve mice or tumor-bearing mice do not possess the suppressive function; but they become suppressive after culture corroborated by the fact that MDSC from tumor tissue rather than spleen possess the suppressive characteristics. In regaining of suppressive function was because these MDSC differentiated into suppressive macrophages ($F4/80^+$) [35 administration recovered their suppressive functionalities including the expression of Arg1, the suppression of the proli as suppression of the development of VLS after the adoptive transfer (Fig. 8A–8D).

Whether resveratrol can induce the differentiation of the suppressive macrophages or how resveratrol recoveres the s examined the interaction between resveratrol and MDSC. AhR functions as one the main receptors for resveratrol [23 could induce the expression of AhR in the MDSC while IL-2 could not. Interestingly, IL-2 and resveratrol co-treatment This could be because resveratrol is more effective in activated cells than unactivated cells [30]. We noted that the ex expression level of Arg1 and suppression functionality of MDSC. IL-MDSC showed the lowest level of AhR whereas F assume that the signals from AhR may be critical for MDSCs to recover their function. The signals from AhR-resveratr derived signals that induce the suppressive function of MDSCs. Further studies are needed to clarify this hypothesis.

Resveratrol has been shown to possess chemopreventive activities such as suppression of tumor cell proliferation, inc enhancement of chemosensitization of tumor cells [21], [22], [24], [25]. It is known that resveratrol can directly induce chemoresistant B16 melanoma that leads to tumor regresson; meanwhile, resveratrol makes melanomas more suscep Our results showed: (1) resveratrol significantly enhanced the expression of FoxO1 gene of melanoma (Fig. 9A-9C). (susceptibility of melanoma to LAK cytotoxicity (Fig. 9D). It is known that FoxO1 controls the tumor growth; the expres

triggers the ultimate death of tumor [31], [32]. We propose that the above two effects of resveratrol might compensat the tumor infiltrating T lymphocytes so as to promote anti-tumor immunity.

The response to immunotherapy with HDIL-2 in patients with metastatic RCC and melanoma is about 15% to 23%. Th treatment, such as induction of Tregs, could be part of the reason why HDIL-2 remains less effective [1], [2], [3], [4], | induction of MDSC is also an important factor in affecting the results of the treatment. The functional status of immune responsiveness of the therapy in patients. The clinical evidence is needed to further elucidate such an issue. Resverat HDIL-2 therapy by suppressing endothelial damage while enhancing sensitivity of tumor killing. In our previous studies, can show differential susceptibility to LAK lysis based on the expression of CD44 variant isoforms [27]. Therefore, res of CD44 variants in melanoma cells, ECs, LAK, Treg, and MDSCs that contributes to the differential role of resveratro Further studies are necessary to address the role of resveratrol in modulating CD44 isoform expression.

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Author Contributions

Conceived and designed the experiments: HG. Performed the experiments: HG NPS UPS. Analyzed the data: HG PSI tools: PSN MN. Wrote the paper: HG PSN MN.

References

1. Dutcher J (2002) Current status of interleukin-2 therapy for metastatic renal cell carcinoma and metastatic me Find this article online
2. Schwartz RN, Stover L, Dutcher J (2002) Managing toxicities of high-dose interleukin-2. Oncology (Williston P
3. Baluna R, Vitetta ES (1997) Vascular leak syndrome: a side effect of immunotherapy. Immunopharmacology 3
4. McDermott DF (2009) Immunotherapy of metastatic renal cell carcinoma. Cancer 115: 2298–2305. Find this a
5. Rosenberg SA, Mule JJ, Spiess PJ, Reichert CM, Schwarz SL (1985) Regression of established pulmonary m by the systemic administration of high-dose recombinant interleukin 2. J Exp Med 161: 1169–1188. Find this a
6. Cotran RS, Pober JS, Gimbrone MA Jr, Springer TA, Wiebke EA, et al. (1988) Endothelial activation during int mechanism for the vascular leak syndrome. J Immunol 140: 1883–1888. Find this article online
7. Baluna R, Rizo J, Gordon BE, Ghetie V, Vitetta ES (1999) Evidence for a structural motif in toxins and interleu endothelial cells and initiating vascular leak syndrome. Proc Natl Acad Sci U S A 96: 3957–3962. Find this arti
8. Assier E, Jullien V, Lefort J, Moreau JL, Di Santo JP, et al. (2004) NK cells and polymorphonuclear neutrophils vascular leak syndrome. J Immunol 172: 7661–7668. Find this article online
9. Damle NK, Doyle LV (1989) IL-2-activated human killer lymphocytes but not their secreted products mediate i endothelial monolayers. Implications for vascular leak syndrome. J Immunol 142: 2660–2669. Find this article
10. Rosenstein M, Ettinghausen SE, Rosenberg SA (1986) Extravasation of intravascular fluid mediated by the sy 2. J Immunol 137: 1735–1742. Find this article online
11. Lentsch AB, Miller FN, Edwards MJ (1999) Mechanisms of leukocyte-mediated tissue injury induced by interle 243–248. Find this article online
12. Melencio L, McKallip RJ, Guan H, Ramakrishnan R, Jain R, et al. (2006) Role of CD4(+)CD25(+) T regulatory Immunol 18: 1461–1471. Find this article online
13. Gabrilovich DI, Nagaraj S (2009) Myeloid-derived suppressor cells as regulators of the immune system. Nat R
14. Greten TF, Manns MP, Korangy F (2011) Myeloid derived suppressor cells in human diseases. Int Immunopha
15. Hoechst B, Voigtlaender T, Ormandy L, Gamrekelashvili J, Zhao F, et al. (2009) Myeloid derived suppressor c hepatocellular carcinoma via the NKp30 receptor. Hepatology 50: 799–807. Find this article online
16. Li H, Han Y, Guo Q, Zhang M, Cao X (2009) Cancer-expanded myeloid-derived suppressor cells induce anerg

TGF-beta 1. J Immunol 182: 240–249. Find this article online
17. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI (2004) Antigen-specific inhibition of CD8+ T cell response mediated by reactive oxygen species. J Immunol 172: 989–999. Find this article online
18. Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, et al. (2007) Altered recognition of antigen is a mecha Med 13: 828–835. Find this article online
19. Elies J, Cuinas A, Garcia-Morales V, Orallo F, Campos-Toimil M (2011) Trans-resveratrol simultaneously incre release in human endothelial cells. Mol Nutr Food Res 55: 1237–1248. Find this article online
20. Wang H, Yang YJ, Qian HY, Zhang Q, Xu H, et al. (2011) Resveratrol in cardiovascular disease: what is know
21. Namasivayam N (2011) Chemoprevention in experimental animals. Ann N Y Acad Sci 1215: 60–71. Find this a
22. Gupta SC, Kannappan R, Reuter S, Kim JH, Aggarwal BB (2011) Chemosensitization of tumors by resveratrol article online
23. Singh NP, Singh UP, Hegde VL, Guan H, Hofseth L, et al. (2011) Resveratrol (trans-3,5,4′-trihydroxystilbene) s apoptosis involving reciprocal regulation of SIRT1 and NF-kappaB. Mol Nutr Food Res 55: 1207–1218. Find th
24. Osmond GW, Augustine CK, Zipfel PA, Padussis J, Tyler DS (2012) Enhancing Melanoma Treatment with Res article online
25. Gatouillat G, Balasse E, Joseph-Pietras D, Morjani H, Madoulet C (2010) Resveratrol induces cell-cycle disru melanoma. J Cell Biochem 110: 893–902. Find this article online
26. Guan H, Nagarkatti PS, Nagarkatti M (2007) Blockade of hyaluronan inhibits IL-2-induced vascular leak syndro treatment for metastatic melanoma. J Immunol 179: 3715–3723. Find this article online
27. McKallip RJ, Fisher M, Do Y, Szakal AK, Gunthert U, et al. (2003) Targeted deletion of CD44v7 exon leads to cell killing mediated by interleukin-2-activated cytolytic lymphocytes. J Biol Chem 278: 43818–43830. Find this
28. Mustafa A, McKallip RJ, Fisher M, Duncan R, Nagarkatti PS, et al. (2002) Regulation of interleukin-2-induced using hyaluronic acid and anti-CD44 antibodies. J Immunother 25: 476–488. Find this article online
29. Rafi AQ, Zeytun A, Bradley MJ, Sponenberg DP, Grayson RL, et al. (1998) Evidence for the involvement of Fa vascular leak syndrome. J Immunol 161: 3077–3086. Find this article online
30. Singh NP, Hegde VL, Hofseth LJ, Nagarkatti M, Nagarkatti P (2007) Resveratrol (trans-3,5,4′-trihydroxystilben encephalomyelitis, primarily via induction of apoptosis in T cells involving activation of aryl hydrocarbon recepto 1508–1521. Find this article online
31. Zhao Y, Yang J, Liao W, Liu X, Zhang H, et al. (2010) Cytosolic FoxO1 is essential for the induction of autopha Biol 12: 665–675. Find this article online
32. Medema RH, Jaattela M (2010) Cytosolic FoxO1: alive and killing. Nat Cell Biol 12: 642–643. Find this article
33. Singh UP, Singh NP, Singh B, Hofseth LJ, Taub DD, et al. (2012) Role of resveratrol-induced CD11b(+) Gr-1(+ the reduction of CXCR3(+) T cells and amelioration of chronic colitis in IL-10(-/-) mice. Brain Behav Immun 26:
34. Yang Y, Paik JH, Cho D, Cho JA, Kim CW (2008) Resveratrol induces the suppression of tumor-derived CD4+ Immunopharmacol 8: 542–547. Find this article online
35. Watanabe S, Deguchi K, Zheng R, Tamai H, Wang LX, et al. (2008) Tumor-induced CD11b+Gr-1+ myeloid cel draining lymph nodes. J Immunol 181: 3291–3300. Find this article online
36. Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, et al. (2008) Identification of d suppressor cell subpopulations with distinct T cell-suppressive activity. Blood 111: 4233–4244. Find this article
37. Donskov F, Bennedsgaard KM, Hokland M, Marcussen N, Fisker R, et al. (2004) Leukocyte orchestration in bl based immunotherapy in metastatic renal cell carcinoma. Cancer Immunol Immunother 53: 729–739. Find this
38. Rodriguez PC, Ernstoff MS, Hernandez C, Atkins M, Zabaleta J, et al. (2009) Arginase I-producing myeloid-de are a subpopulation of activated granulocytes. Cancer Res 69: 1553–1560. Find this article online
39. Kusmartsev S, Gabrilovich DI (2005) STAT1 signaling regulates tumor-associated macrophage-mediated T ce this article online
40. Narita Y, Wakita D, Ohkur T, Chamoto K, Nishimura T (2009) Potential differentiation of tumor bearing mouse suppressor macrophages and immunostimulatory dendritic cells. Biomed Res 30: 7–15. Find this article online