Resveratrol counters systemic lupus erythematosus-associated atherogenicity by normalizing cholesterol efflux

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Abstract

Resveratrol is a bioactive molecule used in dietary supplements and herbal medicines and consumed worldwide. Numerous investigations by our group and others have indicated cardioprotective and anti-inflammatory properties of resveratrol. The present study explored potential atheroprotective actions of resveratrol on cholesterol efflux in cultured human macrophages exposed to plasma from systemic lupus erythematosus (SLE) patients. These results were confirmed in ApoE^{-/-}Fas^{-/-} double knockout mice, displaying a lupus profile with accelerated atherosclerosis. Resveratrol treatment attenuated atherosclerosis in these mice. THP-1 human macrophages were exposed to 10% pooled or individual plasma from patients who met diagnostic criteria for SLE. Expression of multiple proteins involved in reverse cholesterol transport (ABCA1, ABCG1, SR-B1, and cytochrome P450 27-hydroxylase) was assessed using QRT-PCR and Western blotting techniques. Ten-week-old ApoE^{-/-}Fas^{-/-} double knockout mice ($n=30$) were randomly divided into two equal groups of 15, one of which received 0.01% resveratrol for 10 consecutive weeks. Atherosclerosis progression was evaluated in murine aortas. Bone marrow-derived macrophages (BMDM) were cultured and expression of cholesterol efflux proteins was analyzed in each group of mice. Our data indicate that inhibition of cholesterol efflux by lupus plasma in THP-1 human macrophages is rescued by resveratrol. Similarly, administration of resveratrol in a lupus-like murine model reduces plaque formation *in vivo* and augments cholesterol efflux in BMDM. This study presents evidence for a beneficial role of resveratrol in atherosclerosis in the specific setting of SLE. Therefore, resveratrol may merit investigation as an additional resource available to reduce lipid deposition and atherosclerosis in humans, especially in such vulnerable populations as lupus patients.

Keywords: Systemic lupus erythematosus, atherosclerosis, cholesterol transport, bone marrow-derived macrophages

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Introduction

Premature atherosclerotic cardiovascular disease (ASCVD) frequently complicates systemic lupus erythematosus (SLE). Cardiovascular complications related to atherosclerosis among patients with SLE contribute to their disability and death.^{1,2} Previous work by our group has demonstrated that plasma from patients with SLE impairs cholesterol flux in cultured human macrophages.^{3,4} Here, we explore the effect of resveratrol on cholesterol transport in THP-1 human macrophages, a well-accepted model for atherosclerosis⁵ in the presence of plasma from individuals with SLE. For further insight into *in vivo* effects, we studied resveratrol effects in a murine model of lupus⁶ with atherosclerosis.

Cholesterol balance in macrophages depends on three major pathways: cholesterol uptake by designated receptors, intracellular catabolism, and efflux of cholesterol to

extracellular acceptors. Abnormal lipid uptake into the cells is mostly accomplished by the scavenger receptors: CD36, scavenger receptor (SR)-A1, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), and CXC chemokine ligand (CXCL)16.^{7–9} Intracellular cholesterol processing is represented by the cytochrome P450 27-hydroxylase, an enzyme that converts cholesterol to oxysterols that are polar and thus more readily extracted from cells than cholesterol.¹⁰ Cholesterol efflux from cells of the arterial wall to extracellular acceptors involves the ATP-binding cassette transporters (ABC)A1 and ABCG1.^{11,12} Overall, expression of the proteins mentioned above comprises a cholesterol transport gene profile which reflects cellular influx, processing, and elimination of cholesterol and its derivatives.

Cholesterol influx and lipid uptake into macrophages by SRs is a highly spontaneous and unregulated process.

In contrast, expression of proteins involved in cholesterol efflux is carefully controlled and subject to manipulation with multiple pharmacological agents. One candidate for use in controlling cholesterol outflow is resveratrol (3,5,4'-trihydroxy-trans-stilbene), a bioactive molecule used in dietary supplements, already reported to display cardioprotective and anti-inflammatory properties.^{13,14}

Here we present additional data to support the use of resveratrol in blocking atheroma-promoting effects of the lupus milieu in humans and in a murine model. We report that resveratrol can protect THP-1 human macrophages from pro-atherogenic effects of SLE plasma. Moreover, in ApoE^{-/-}Fas^{-/-} double knockout mice with atherosclerosis and lupus-like disease, resveratrol reduces plaque formation in the aorta.

Materials and methods

Cell culture and experimental conditions

Resveratrol (Sigma-Aldrich, St. Louis, MO) stock solutions were prepared in ethanol.

THP-1 monocytes were grown at 37°C in a 5% CO₂ atmosphere in RPMI 1640.⁸ To facilitate differentiation into macrophages, THP-1 monocytes were treated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 24 h at 37°C. Upon PMA removal, the macrophages were cultured for another 24 h prior to treatment. THP-1 macrophages were incubated in 12-well plates for 18 h in RPMI medium under the following eight conditions: (1) 10% fetal calf serum; (2) 10% pooled normal human plasma (Innovative Research, Novi, MI); (3) 10% pooled plasma from 10 healthy control subjects (HP); (4) 10% pooled plasma from 10 SLE patients; (5) 10% pooled HP plasma + solvent control (EtOH); (6) 10% pooled HP plasma + 10 μM resveratrol; (7) 10% pooled SLE plasma + EtOH; (8) 10% pooled SLE plasma + 10 μM resveratrol. No fetal calf serum was added to cells incubated in human plasma.

For mouse bone marrow-derived macrophage (BMDM) studies, femurs were dissected and cell suspensions obtained by flushing femurs and tibias with sterile phosphate-buffered saline (PBS). After dispersion by passing the marrow through an 18 gauge needle several times, cells were centrifuged at 500 × g for 5 min. Cells were then resuspended in R10 medium containing 15% L929-cell conditioned medium, plated in 100 mm culture dishes, and incubated at 37°C in a 5% CO₂ atmosphere for seven days. Medium was aspirated and replaced on days 3 and 5. BMDMs were generated by culturing cells for seven days with macrophage colony-stimulating factor (MCSF).¹⁵ Cells from all genotypes of mice: C57BL/6J, ApoE^{-/-}, and ApoE^{-/-}Fas^{-/-} were incubated with or without solvent control (EtOH) or 10 μM resveratrol and subjected to total RNA isolation, protein extraction, and cholesterol efflux analysis. To confirm a beneficial effect of resveratrol *in vivo*, BMDMs were collected in ApoE^{-/-}Fas^{-/-} mice before and after administration of resveratrol in drinking water and subjected to the assays described above.

Subject inclusion and exclusion criteria

Human subject studies were performed under protocol #08310 approved by the Institutional Review Board of Winthrop University Hospital. Written informed consent was obtained from all enrolled subjects of the study: 10 SLE patients between age 30 and 50 years, males and females, and 10 healthy controls (HC) sex- and age-matched to the patient group. The mean duration of disease for SLE patients was 10–14 years. Patients had to fulfill the 1982 revised criteria of the American College of Rheumatology for classification of SLE.¹⁶ Patients with previous documentation of a diagnosis of a connective tissue disorder other than SLE were excluded. Patients had no statin treatment in the prior three months.

Generation of ApoE^{-/-}Fas^{-/-} mice and resveratrol administration

ApoE^{-/-} and Fas^{-/-} (lpr/lpr) mice on the B6 background were purchased from the Jackson Laboratories (Bar Harbor, ME). Single knockout mice were intercrossed and backcrossed to the ApoE^{-/-} parents to produce three groups of mice with the following genotypes: ApoE^{-/-}Fas^{+/+}, ApoE^{-/-}Fas^{+/-}, and ApoE^{-/-}Fas^{-/-}. DNA was extracted from tails using the Qiagen DNeasy Tissue Kit. Genotyping of the wild type versus the apoE knockout allele¹⁷ and the lpr allele¹⁸ was performed as described. All mice were fed a regular chow diet (Harlan Teklad TD.91354) and maintained in a temperature-controlled room with a 12 h light/dark cycle.

Ten-week-old ApoE^{-/-}Fas^{-/-} double knockout mice (*n* = 30) were randomly divided into two groups. Mice in the first group (*n* = 15) received 0.01% resveratrol in the drinking water for 10 consecutive weeks. Resveratrol was first dissolved in 0.4 mL of absolute ethanol and added to 100 mL of drinking water. The control group of ApoE^{-/-}Fas^{-/-} mice (*n* = 15) received 0.4% ethanol vehicle in the same amount of drinking water, administered until age 20 weeks. At the end of the treatment one mouse from control group had died. All mice were maintained on a regular chow diet and had free access to drinking solutions. The daily consumption of resveratrol was 0.3–0.4 mg/mouse in the treatment group.

All mice were used in accordance with National Institutes of Health guidelines. All protocols, including handling, husbandry, euthanasia, and resveratrol administration, were approved by Winthrop University Hospital-IACUC (protocol AR#4 and AR#5).

Assessment of atherosclerotic lesions in the murine aorta

After 10 weeks of vehicle or resveratrol administration, mice were euthanized with CO₂ and blood was collected by heart puncture after sacrifice. The main circulation was cleared by perfusion of the left ventricle with 0.9% saline until right ventricle washout was clean. The entire aorta, attached to the heart and lungs, up to the iliac bifurcation was excised. Collected aorta specimens were then fixed in neutral buffered 10% formalin (Poly Scientific, Bay Shore, NY) from 24 h to one week. Adventitial connective tissue

with fat was removed and aorta with attached heart and lungs was mounted en face and embedded in paraffin.

Embedded aorta specimens were sectioned at 5 μ m along their entire length. Three sets of histological sections were obtained for each animal. For each of the three sets, one of the sections was stained with hematoxylin and eosin (H&E stain, Sigma-Aldrich, St. Louis, MO). Then each set of histological sections was analyzed to identify the immediate supravalvular aorta, as manifested by the aortic valve and proximal aorta in the section. This site was used because the control group of ApoE^{-/-}/Fas^{-/-} double knockout mice was noted to develop the most profound atherosclerotic lesions in this area. Sections with a greater number and area of intimal involvement in atherosclerotic lesions were stained with Trichrome Stain (Masson) Kit (Sigma-Aldrich, St. Louis, MO). Trichrome staining was used to highlight intimal thickening, identify cholesterol crystals, and intimal and medial collagen deposition.

Section analysis was performed by a trained observer blinded to the treatment status of the mice. Width, length, and surface plaque area were acquired for each identified supravalvular aortic site. Histological image analyzer Nikon digital sight DS-L2 was used for measurement of plaques and making color images at 40X magnification. The atherosclerotic index (AI), defined as the median surface plaque area, was tabulated, and the value was used to represent atherosclerosis progression per animal. This index was used to compare atherosclerosis development between control and resveratrol-treated groups.

RNA isolation and gene expression analysis by QRT-PCR

THP-1 human macrophages and murine BMDM with or without incubation were subjected to isolation of total RNA. Isolation was done with Trizol reagent according to the manufacturer's instructions and dissolved in nuclease-free water. The quantity of total RNA from each condition was measured by absorption at 260 and 280 nm wavelengths by ultraviolet spectrophotometry (Hitachi U2010 spectrophotometer).

QRT-PCR analysis was performed using the FastStart SYBR Green Reagents Kit according to the manufacturer's instructions on the Roche Light Cycler 480 (Roche Applied Science, Indianapolis, IN). cDNA was copied from 1 μ g of total RNA using Murine Leukemia Virus reverse transcriptase primed with oligo dT. Equal amounts of cDNA were taken from each reverse transcription reaction mixture for real-time PCR amplification using gene-specific primers for 27-hydroxylase, ABCA1 and ABCG1, SR-B1, as well as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1).

QRT-PCR reactions were done in triplicate. To correct for differences in cDNA load among samples, the target PCRs were normalized to a reference PCR involving the endogenous housekeeping gene GAPDH. The fold change in expression level of target gene relative to the GAPDH at various time points was calculated using $2^{-\Delta\Delta CT}$ method.

Table 1 The list of specific primers used for QRT-PCR

Gene	Primer
ABCA1	F 5'-GAAGTACATCAGAACATGGGC-3' R 5'-GATCAAAGCCATGGCTGTAG-3'
ABCG1	F 5'-CAGGAAGATTAGACACTGTGG-3' R 5'-GAAAGGGGAATGGAGAGAAG-3'
27-hydroxylase	F 5'-AAGCGATACCTGGATGGTTG-3' R 5'-TGTTGGATGTCGTGTCCACT-3'
SCRB1	F 5'-GGTCCCTGTCATCTGCCAA-3' R 5'-CTCCTTATCCTTTGACCCCTT-3'
GAPDH	F 5'-ACCATCATCCCTGCCTCTAC-3' R 5'-CCTGTTGCTGTAGCCAAAT-3'

Non-template controls were included for each primer pair to check for significant levels of any contaminants. A melting curve analysis was performed to assess the specificity of the amplified PCR products.

Protein extraction and Western blot analysis

Cellular extracts were prepared with Lysis Kit—RIPA Buffer (ProteinSimple, Santa Clara, CA). The immunoreactive proteins were detected using a Wes Assay kit (ProteinSimple, Santa Clara, CA).

Rabbit anti-human ABCA1 (sc-20794) (Santa Cruz, CA) and rabbit anti-human ABCG1 (ab-36969) (Abcam Inc., Cambridge, MA) were used as primary antibodies for detection of ABCA1 and ABCG1, respectively. As a loading control, GAPDH was detected using rabbit anti-human GAPDH antibody (ab9485).

Quantization of detected proteins and image preparation were performed with Compass Software (ProteinSimple, Santa Clara, CA).

Cholesterol efflux analysis

Cholesterol efflux was analyzed in THP-1 macrophages in the presence of HP or SLE plasma with or without solvent control or 10 μ M resveratrol. Similarly, naïve BMDM from C57BL/6J wild-type mice, ApoE^{-/-} and ApoE^{-/-}Fas^{-/-} mice with and without solvent control or 10 μ M resveratrol, and in BMDM from ApoE^{-/-}Fas^{-/-} mice before and after resveratrol administration were subjected to Amplex Red Cholesterol Assay. The Assay Kit (Molecular Probes, Eugene, OR) was used according to the manufacturer's protocol. Performing reactions in the presence and absence of cholesterol esterase, total (TC) and free (FC) cholesterol were analyzed. Cholesterol esters (CE) were estimated as the difference between TC and FC and CE/FC ratio was calculated.

Data analysis

Statistical analysis was performed using Graphpad Prism, version 5.01 (GraphPad Software, San Diego, CA) and SAS version 9.3 (SAS Institute, Cary, NC). All *in vitro* data were analyzed by one-way analysis of variance, and pairwise multiple comparisons were made between various treatment conditions using a Bonferroni correction.

Figure 1 Resveratrol restores cholesterol efflux in THP-1 human macrophages exposed to plasma from SLE patients. THP-1 macrophages were incubated in the presence of 10% pooled plasma from 10 control subjects (HP) or 10 SLE subjects with or without solvent control (EtOH) or 10 μ M resveratrol (Resv), as indicated in methods. Following incubation, total RNA isolated from cells of each condition was reverse transcribed and amplified by QRT-PCR with GAPDH message as an internal standard. Gene expression levels were graphed as relative mRNA expression with mean of HP set at 100% (a). Gene expression was confirmed by Western blot (b). Data represent the mean for three independent experiments. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ versus macrophages exposed to HP plasma, ## $P < 0.01$ versus cells exposed to SLE plasma. The presence of solvent did not affect expression of the targeted proteins. Therefore, for demonstrational purposes solvent condition was not included in the graph. Cholesterol efflux assay was performed in the same cells plated and treated simultaneously (c). * $P < 0.05$ versus cells exposed to HC plasma; # $P < 0.05$ versus cell with SLE plasma alone. (A color version of this figure is available in the online journal.)

The AI values used in the *in vivo* murine study analysis were based on the mouse-level median AI values computed across all observed plaques. Due to the skewed nature of the AI (a moderate proportion of zeroes due to no developed plaques in some mice, as well as extremely large AI values in other mice), we approached the analysis in several ways to verify the robustness of our result. First, we used a Wilcoxon Mann-Whitney test to compare the distribution of the median AI in treated versus control mice. We then categorized AI values into deciles and fit an ordinal logistic regression model to estimate the group effect on the probability of higher deciles. We also compared plaque counts in treated versus control mice via zero-inflated Poisson models. Finally, litter-level median AI values were summarized and compared between groups. Two-sided p -values less than 0.05 were regarded as statistically significant.

Results

Resveratrol rescues cholesterol efflux in THP-1 human macrophages exposed to SLE patient plasma

The presence of SLE plasma markedly diminished expression of cholesterol efflux proteins in THP-1 macrophages

(Figure 1(a)). Thus, 10% SLE plasma suppressed ABCA1 and ABCG1 expression in macrophages by $58.3 \pm 15.5\%$ ($n = 3$, $P < 0.001$) and $48.3 \pm 18.7\%$ ($n = 3$, $P < 0.01$), respectively, below HP (set at 100%). 27-hydroxylase message fell by $36.2 \pm 12.8\%$ versus HP ($n = 3$, $P < 0.05$). The presence of the solvent—EtOH did not significantly alter expression of these proteins. When THP-1 macrophages were incubated with SLE plasma + resveratrol, efflux proteins were restored to the level in cells exposed to HP. Results of the gene expression analysis were confirmed by Western blot (Figure 1(b)).

Beneficial effect of resveratrol on the expression of cholesterol efflux proteins was confirmed by cholesterol efflux analysis in THP-1 human macrophages (Figure 1(c)). Cholesterol mass was quantitated fluorometrically using the Amplex Red Cholesterol Assay Kit. We observed that resveratrol augmented the ability of THP-1 macrophages to remove cholesterol from cells to the medium. Thus, in cells exposed to SLE plasma, efflux was decreased and resulted in accumulation of intracellular cholesterol (mainly ChE) versus cells exposed to HP ($n = 3$, $P < 0.05$) (Figure 1(c)). The level of FC was not significantly affected. Therefore, cells incubated in the presence of SLE plasma display an

Figure 2 Vascular atherosclerotic lesions in 20-week-old ApoE^{-/-}Fas^{-/-} mice with and without administration of resveratrol in the drinking water. Representative images of 5 μm thick sections taken of the mouse aorta obtained from the evaluated cohort ($n = 14$) in control group of ApoE^{-/-}Fas^{-/-} mice and mice ($n = 15$) after resveratrol administration. Images taken in the area of whole embedded aorta X100 (a) or lesions within the aortic sinus region (b). The sections were stained with Trichrome stain highlighting progression of atherosclerosis with accumulation of lipids (white cholesterol crystals) and vascular fibrosis with increase of extracellular matrix (blue). The muscle tissue is stained red. The distribution and median value of the atherosclerotic index (AI) was calculated and graphed (c) WMW ($p < 0.001$) versus control group. (A color version of this figure is available in the online journal.)

increase in the intracellular ratio of ChE to FC versus cells incubated with HP. ChE are much less polar than FC and are major constituents of the fatty lesions in atherosclerotic plaques. The presence of resveratrol abolished the effect of SLE plasma on cholesterol efflux, decreasing the ChE/FC ratio and maintaining efflux similar to cells incubated with HP.

Resveratrol administration in ApoE^{-/-} Fas^{-/-} double knockout lupus mice reduces atherosclerotic plaque formation

Atherosclerotic plaques from the control untreated group of double knockout ApoE^{-/-}Fas^{-/-} mice displayed a more advanced stage of atheroma progression. Eighty percent of mice in the control group demonstrated simultaneous acceleration of atherosclerosis and fibrosis (Figure 2(a)). The majority of plaques consisted of fatty streaks with accumulation of cholesterol crystals (white) and collagen (blue). In contrast, the resveratrol-treated group of mice exhibited predominantly early stages of atherosclerotic plaques from fatty dots (29% of treated mice) to the first evidence of collagen fibrosis (Figure 2(b)). In only two mice (13%) from the resveratrol-treated group, atherosclerotic plaques were as developed as those in the control group. Forty-three percent of double knockout mice receiving resveratrol did not develop plaques at all.

The number of atherosclerotic plaques from the control untreated group of double knockout ApoE^{-/-}Fas^{-/-} mice also was observed to be more frequent compared to those

mice given resveratrol; however, this difference was not statistically significant. The distribution and median value of the AI calculated was observed to be markedly lower in mice given resveratrol than for ApoE^{-/-}Fas^{-/-} mice fed regular diet (median AI: 1094 μm² versus 52,827 μm², respectively; WMW $p < 0.001$) (Figure 2(c)). Examination of the ordinal logistic regression model confirmed this result: the odds of a mouse in the control group being in a higher AI decile was 20 times that of a resveratrol-treated mouse (lower limit of 95% CI for the odds ratio: 3.8; $p < 0.001$). Litter-level analysis, comparing median litter AI values in resveratrol treated versus control ApoE^{-/-}Fas^{-/-} mice was consistent with the mouse-level analysis (WMW $p = 0.01$).

Resveratrol enhances cholesterol efflux in BMDM from C57BL/6J, ApoE^{-/-}, ApoE^{-/-}Fas^{-/-} mice

We have explored the effect of resveratrol on cholesterol efflux in BMDM from C57BL/6J, ApoE^{-/-}, and ApoE^{-/-}Fas^{-/-} mice (Figure 3). ApoE^{-/-} and ApoE^{-/-}Fas^{-/-} mice displayed a twofold reduction in mRNA level of ABCA1 and ABCG1 ($n = 3$, $P < 0.01$) compared to C57BL/6J wild-type mice (set at 1.0). When cells were cultured with solvent control, no significant changes in the expression of the targeted proteins were detected. In cells derived from different murine genotypes upon exposure to resveratrol, significant augmentation of cholesterol efflux proteins was observed (Figure 3). The mRNA level of ABCA1, ABCG1, and 27-hydroxylase was increased in the

Figure 3 Cholesterol efflux in BMDM from 20-week-old C57BL/6J, ApoE^{-/-}, ApoE^{-/-} Fas^{-/-} ± resveratrol (Resv). Total RNA were isolated and level of ABC transporters, SR-B1 and 27-hydroxylase were analyzed using QRT-PCR (***P* < 0.01; ****P* < 0.001 versus C57BL/6J 1.0; ##*P* < 0.01 versus untreated cells)

presence of resveratrol to 1.58 ± 0.054 , 1.28 ± 0.048 , and to 1.44 ± 0.17 -fold in C57BL/6J, respectively. Culturing BMDM from ApoE^{-/-} and ApoE^{-/-} Fas^{-/-} in the presence of resveratrol doubled expression of cholesterol efflux proteins, raising their levels to those observed in C57BL/6J ($n=3$, $P < 0.01$). ABCA1 and 27-hydroxylase protein expressions were upregulated twofold in all analyzed cells ($n=3$, $P < 0.01$) (Figure 3). In contrast, expression of SR-B1 was not significantly changed in the presence of resveratrol.

These experiments reflect a response similar to that in human macrophages.

Described alterations in the expression of the cholesterol efflux proteins correlated with an increase in accumulation of ChE in BMDM from ApoE^{-/-} and ApoE^{-/-} Fas^{-/-} mice (reaching $7.74 \pm 0.53 \mu\text{g/mL}$ in ApoE^{-/-} BMDM and $7.02 \pm 0.11 \mu\text{g/mL}$ in ApoE^{-/-} Fas^{-/-} versus $3.2 \pm 0.5 \mu\text{g/mL}$ in C57BL/6J, $n=4$, $P < 0.05$). When BMDMs were exposed to $10 \mu\text{M}$ resveratrol, the level of ChE in ApoE^{-/-} BMDM decreased to $5.7 \pm 0.16 \mu\text{g/mL}$ and in ApoE^{-/-} Fas^{-/-} to $6.05 \pm 0.11 \mu\text{g/mL}$ (Figure 4). No significant changes were noted in the level of FC.

Resveratrol administration in apoE^{-/-} Fas^{-/-} double knockout lupus mice augments cholesterol efflux in BMDM

Next, we analyzed changes in expression of cholesterol efflux proteins in BMDM of ApoE^{-/-} Fas^{-/-} mice before and after resveratrol administration (Figure 5). ApoE^{-/-} Fas^{-/-} mice displayed a twofold reduction in mRNA level of ABCA1 and ABCG1 ($n=3$, $P < 0.01$) compared to the same age and gender C57BL/6J wild-type mice (set at 1). Resveratrol administration in ApoE^{-/-} Fas^{-/-} mice

augments expression of these genes twofold ($n=3$, $P < 0.05$) (Figure 5(a)). The level of 27-hydroxylase was upregulated 2.34 times after resveratrol administration as well. Gene expression analysis was confirmed by Western blot (Figure 5(b)). Resveratrol administration did not affect the expression of SR-B1 in ApoE^{-/-} Fas^{-/-} mice.

Discussion

ASCVD is a characteristic feature of SLE and currently accounts for 20–30% of deaths in SLE patients.¹⁹ In SLE, the risk of being affected by atherosclerotic coronary artery disease is 4–8 times higher than in the normal population and the lesions develop in young premenopausal women in whom this disease is otherwise rare.²⁰ Atherosclerosis contributes to approximately 30% of deaths in SLE patients and about 30% of SLE patients have subclinical atherosclerosis. Coronary artery atherosclerosis risk in SLE cannot be predicted by Framingham risk factors or disease activity markers. ASCVD in SLE cannot be fully explained by traditional Framingham risk factors alone. The inadequacy of classic risk factors as predictive tools compromises the ability of the treating physician to determine which patients are most vulnerable to atherosclerosis.^{21–23} Treatment and prevention of ASCVD in these patients is difficult. In the Lupus Atherosclerosis Prevention Study, Petri *et al.*²⁴ showed that the HMG CoA reductase inhibitor atorvastatin failed to reduce subclinical measures of atherosclerosis or disease activity over two years in SLE patients. Previous work by our group and others has demonstrated that autoimmune rheumatic diseases provoke cellular changes in expression of genes responsible for cholesterol flux.^{25–27} This points to a direct

Figure 4 Cholesterol efflux assay in BMDM from (a) C57BL/6 J, (b) ApoE^{-/-}, (c) ApoE^{-/-}Fas^{-/-}, (d) C57BL/6 J+Resv, (e) ApoE^{-/-}+Resv, (f) ApoE^{-/-}Fas^{-/-}+ Resv reflecting changes in CE/FC ratio. **P* < 0.05 versus C57BL/6 J; #*P* < 0.05 versus untreated corresponding BMDM

link between cholesterol metabolism in the artery and immune function.

In light of the inflammatory nature of the lupus milieu and the ineffectiveness of statins as ASCVD treatment in this population, we explored here the utility of the polyphenol resveratrol in combating abnormalities in cholesterol export function in human macrophages exposed to SLE plasma. This study has a number of limitations, including its *in vitro* design and use of plasma samples from a small number of lupus subjects from the Northeastern United States. While these patients may not be generalized to a larger population, this was a study of mechanism, not a clinical study and the atherogenic properties of their plasma were consistent with our findings in multiple previous experiments.^{3,4} Resveratrol, found in a variety of edible plants, including grapes, berries, and peanuts, has been studied extensively for its cardioprotective properties.²⁸ Despite many beneficial effects in animal models,^{29,30} human efficacy studies are limited and

conflicting.³¹⁻³³ The combination of human and murine studies presented here indicates that resveratrol may have specific atheroprotective benefit in the setting of the autoimmune state of SLE. In this situation, resveratrol was able to mitigate pro-atherogenic factors present in the plasma of SLE patients *in vitro* and in a murine model *in vivo*.

In *in vitro* studies resveratrol enhances cholesterol efflux from macrophage via ABCG1 and ABCA1 pathways but does not significantly affect passive efflux. A limitation of cell culture studies is that they are a static system. Therefore, we confirmed benefits of resveratrol administration, observed *in vitro*, conducting similar experiments in a murine study *in vivo*. Utilizing an SLE-like murine model, prone to spontaneous atheroma development, we demonstrate that augmentation of cholesterol efflux by resveratrol leads to a regression of coronary atheroma. Untreated ApoE^{-/-}Fas^{-/-} mice demonstrate more advanced atheroma progression with heightened synthesis of extracellular

Figure 5 Cholesterol transport gene expression in BMDM from 20-week-old ApoE^{-/-}Fas^{-/-} before and after resveratrol (Resv) administration. Total RNA and protein were isolated and level of ABC transporters, SR-B1 and 27-hydroxylase were analyzed using QRT-PCR (a) and Western blot (b) (***P* < 0.01; ****P* < 0.001 versus ApoE^{-/-}Fas^{-/-} mice set at 1.0). (A color version of this figure is available in the online journal.)

matrix macromolecules such as collagen, accumulation of lipids, and cholesterol crystals. Resveratrol prevented lesion maturation with a delay in progression, exhibiting predominantly early stages of atherosclerotic plaques from fatty dots to first evidence of collagen fibrosis.

Salutary effects of resveratrol on cholesterol handling offset the atherogenic influence of SLE. Resveratrol operates as an antiatherogenic agent by preventing lipid overload via augmentation of cholesterol efflux. Since resveratrol has a strong safety profile and is well tolerated, low cost, and can be used in combination with multiple other therapies without contraindication, these findings provide the rationale for a novel therapeutic approach to decrease atherosclerotic cardiovascular consequences of SLE.³⁴ Further *in vivo* studies are indicated for this promising treatment option.

Authors' contribution: All authors participated in the design, interpretation of the studies, and analysis of the data. IV, IT, NMS, MJL, and GKT made substantial

contributions to acquisition of data. IV, IT, MJL, NMS, and ABR conducted the experiments. IV, ABR, GKT, JDL, and SEC wrote the manuscript. All authors read and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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