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Regular Paper

Resveratrol and piceid enhance efferocytosis by increasing the secretion of MFG-E8 in human THP-1 macrophages

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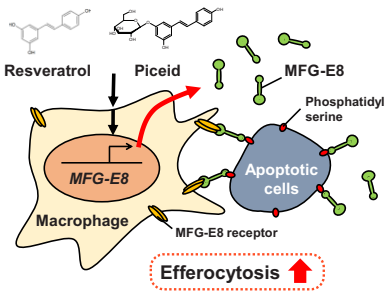
Abstract

The process of apoptotic cell clearance by phagocytes, known as efferocytosis, plays an essential role in maintaining homeostasis. Defects in efferocytosis can lead to inflammatory diseases such as atherosclerosis and autoimmune disorders. Therefore, the maintenance and promotion of efferocytosis are considered crucial for preventing these diseases. In this study, we observed that resveratrol, a representative functional food ingredient, and its glycoside, piceid, promoted efferocytosis in both human THP-1 macrophages differentiated with phorbol 12-myristate 13-acetate and peritoneal macrophages from thioglycolate-elicited mice. Resveratrol and piceid significantly increased mRNA expression and protein secretion of MFG-E8 in THP-1 macrophages. Furthermore, the activation of efferocytosis and the increment in MFG-E8 protein secretion caused by resveratrol or piceid treatment were canceled by MFG-E8 knockdown in THP-1 macrophages. In conclusion, we have demonstrated for the first time that resveratrol and piceid promote efferocytosis through the upregulation of MFG-E8 excretion in human THP-1 macrophages.

Keywords: macrophage, efferocytosis, resveratrol, anti-inflammation, MFG-E8

Graphical abstract



This study revealed that the promotive effect of resveratrol and piceid on efferocytosis by upregulating MFG-E8 excretion in macrophages.

Abbreviations: PS: phosphatidylserine; MFG-E8: milk fat globule-EGF factor 8; Gas6: growth arrest-specific 6; SULT: sulfotransferase; UGT: uridine 5′-diphospho-glucuronosyltransferase; LPS: lipopolysaccharide; FBS: fetal bovine serum; PMA: phorbol 12-myristate 13-acetate; resveratrol-3GA: *trans*-resveratrol 3-O-β-D-glucuronide; CFSE: 5-or 6-(N-succinimidyloxycarbonyl)-fluorescein-3′,6′-diacetate; DMSO: dimethyl sulfoxide

The prompt and efficient clearance of apoptotic cells is essential for maintaining homeostasis, which includes the resolution of inflammation and tissue repairment (Henson 2017). The process of apoptotic cell clearance, called efferocytosis (DeCathelineau and Henson 2003), is carried out by both professional phagocytes such as macrophages (Morioka *et al.* 2018) and dendritic cells (Maschalidi *et al.* 2022), and to a lesser extent by nonprofessional phagocytes like epithelial cells (Ichimura *et al.* 2008). Efferocytosis is conducted *via* 4 steps: (1) the recruitment of the phagocytes by apoptotic cells through find-me signals released in the early

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stages of apoptosis, (2) the recognition of the ligands on apoptotic cells such as phosphatidylserine (PS), known as eat-me signals, by phagocytes through specific receptors, (3) the ingestion of apoptotic cells by phagocytic receptors, and (4) the digestion of ingested apoptotic cells, leading to the production of anti-inflammatory mediators by the efferocytic phagocytes (Morioka, Maueröder and Ravichandran 2019). Macrophages, during efferocytosis, produce anti-inflammatory mediators such as IL-10 and TGF-β, which attenuate the inflammatory response and promote the resolution of inflammation (Fadok *et al.* 1998; Xu *et al.* 2006). On the other hand, defective efferocytosis results in the aberrant accumulation of apoptotic cells, leading to postapoptotic cytolysis, known as secondary necrosis (Sachet, Liang and Oehler 2017). The cellular contents released from these necrotic cells trigger an immune response due to the proinflammatory substances and cell fragments contained in them. If this condition persists chronically, it can lead to inflammatory diseases such as atherosclerosis, inflammatory lung disease, and autoimmune diseases (Boada-Romero *et al.* 2020). Thus, maintaining and promoting the efferocytic activity is an effective strategy for preventing these diseases.

The engulfment of apoptotic cells is not solely facilitated by the find-me and eat-me signals originating from apoptotic cells but is also promoted by opsonins released from phagocytes. Opsonins are soluble, extracellular proteins that enhance the ingestion of pathogen antigens (Marshall *et al.* 2018) and apoptotic cells (Akakura *et al.* 2004) by phagocytes through bridging these targets with phagocytes (Vilalta and Brown 2018). Opsonins promoting the engulfment of apoptotic cells include milk fat globule-EGF factor 8 (MFG-E8), growth arrest-specific 6 (Gas6), calreticulin, and complement C1q and C3b. Notably, MFG-E8 and Gas6 serve as representatives among them. MFG-E8, a glycoprotein secreted by activated macrophages, facilitates efferocytosis by cross-linking PS on apoptotic cells and αvβ3/β5 integrin on phagocytes (Hanayama *et al.* 2002). Gas6, a vitamin K-dependent protein, is identified as a protein inductively expressed in fibroblasts in response to serum depletion and has approximately 43% the same amino acid sequence as protein S, another vitamin K-dependent protein (Manfioletti *et al.* 1993). Activated macrophages also secrete Gas6, promoting efferocytosis by cross-linking PS on apoptotic cells and TAM receptors (Tyro3, Axl, MerTK) on phagocytes (Nagata *et al.* 1996). MFG-E8-deficient mice develop severe autoimmune and inflammatory diseases resembling human systemic lupus erythematosus and atherosclerosis, accompanied by the accumulation of apoptotic cells (Hanayama *et al.* 2004; Ait-Oufella *et al.* 2007). Decreased MFG-E8 has been observed in various diseases including atherosclerosis and Alzheimer's disease (Ait-Oufella *et al.* 2007; Boddaert *et al.* 2007; Dai *et al.* 2014). In a rat model of sepsis, recombinant MFG-E8 reduced the accumulation of apoptotic cells after cecal ligation and puncture, improving the 10-day survival rate (Qiang *et al.* 2011). In obese osteoarthritis mice, more severe cartilage destruction, decreased Gas6 secretion by synovial macrophages, and increased levels of synovial apoptotic cells were observed compared to osteoarthritis mice (Yao *et al.* 2023). Intra-articular injection of recombinant Gas6 in mice restored the efferocytic capacity of macrophages and prevented the progression of obesity-associated osteoarthritis. These reports suggest that deficiencies in opsonins such as MFG-E8 and Gas6 are closely linked to inflammatory diseases, and the up-regulation of these opsonins could be a potential preventive strategy for these diseases.

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a famous dietary polyphenolic compound, is found in red wine and red grape juice (Baur and Sinclair 2006). The hypothesis that resveratrol could explain the "French Paradox," a term describing the epidemiological phenomenon where high milk fat consumption is associated with a higher rate of heart disease mortality, while in France, the rate of heart disease mortality is lower despite the high consumption, has spurred extensive research on its anti-inflammatory and antioxidant effects (St, Cochrane and Moore 1979; Criqui and Ringel 1994). Indeed, resveratrol has been demonstrated to have antioxidative, anti-inflammatory, and immunomodulating properties, along with protective effects in chronic disease models, including cardiovascular disease (Petrovski, Gurusamy and Das 2011), diabetes (Szkudelski and Szkudelska 2015), and neurodegenerative diseases (Tellone *et al.* 2015). Additionally, resveratrol naturally occurs in various plants and fruits, mainly in its glycosylated form known as piceid (also named polydatin or *trans*-resveratrol-3-*O*-β-D-glucoside) (Walle 2011; Gambini *et al.* 2015). As shown in Figure 1, piceid can be metabolized to resveratrol through deglycosylation in the small intestine and liver (Henry-Vitrac *et al.* 2006). Resveratrol undergoes conjugation with sulfate by sulfotransferase and with glucuronate by uridine 5′-diphospho-glucuronosyltransferase, mainly in the small intestine and liver in humans (Springer and Moco 2019). Resveratrol and conjugated metabolites are metabolized by the gut microbiota in the large intestine to generate dihydroresveratrol, 3,4′-dihydroxy-trans-stilbene, and lunularin in humans (Springer and Moco 2019). Piceid has been reported to share many therapeutic benefits with resveratrol, such as protecting cardiomyocytes and inhibiting lipopolysaccharide (LPS)-induced inflammation in renal tissue (Zhang *et al.* 2017; Gu *et al.* 2019; Wu *et al.* 2020). Despite numerous reports indicating that resveratrol and piceid improve the inflammatory state through various mechanisms, whether they activate efferocytosis, which plays an important role in resolving inflammation, remains unclear. In the present study, we investigated the effects of resveratrol and piceid on efferocytic activity and the expression of representative opsonins MFG-E8 and Gas6 in the human monocytic leukemia cell line THP-1, an established human macrophage model *in vitro* (Chanput, Mes and Wichers 2014), and peritoneal macrophages from thioglycolate-elicited mice.

Materials and methods

Reagents

Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Phorbol 12-myristate 13-acetate (PMA), staurosporine, and *trans*-resveratrol-3-*O*-β-D-glucuronide (resveratrol-3GA) were purchased from Cayman Chemical (Ann Arbor, MI, USA). LPS from *Escherichia coli* O127: B8, sulfanilamide, and 3,5-dinitrocatechol were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-or 6-(N-succinimidyloxycarbonyl)-fluorescein-3′,6′-diacetate (CSFE) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ReverTra Ace® qPCR RT Master Mix with gDNA Remover and THUNDERBIRD® SYBR® qPCR Mix were purchased from Toyobo Co., Ltd. (Osaka, Japan). Antibodies against MFG-E8 (sc-377356), Gas6 (ac-376087), and β-Actin (sc-47778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-mouse IgG (AP124P) and goat anti-rabbit IgG (AP132P) were purchased from Merck Millipore (Billerica, MA, USA). Other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Cell culture

The THP-1 cell line was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer Tohoku University, Japan. The human T cell leukemia cell line

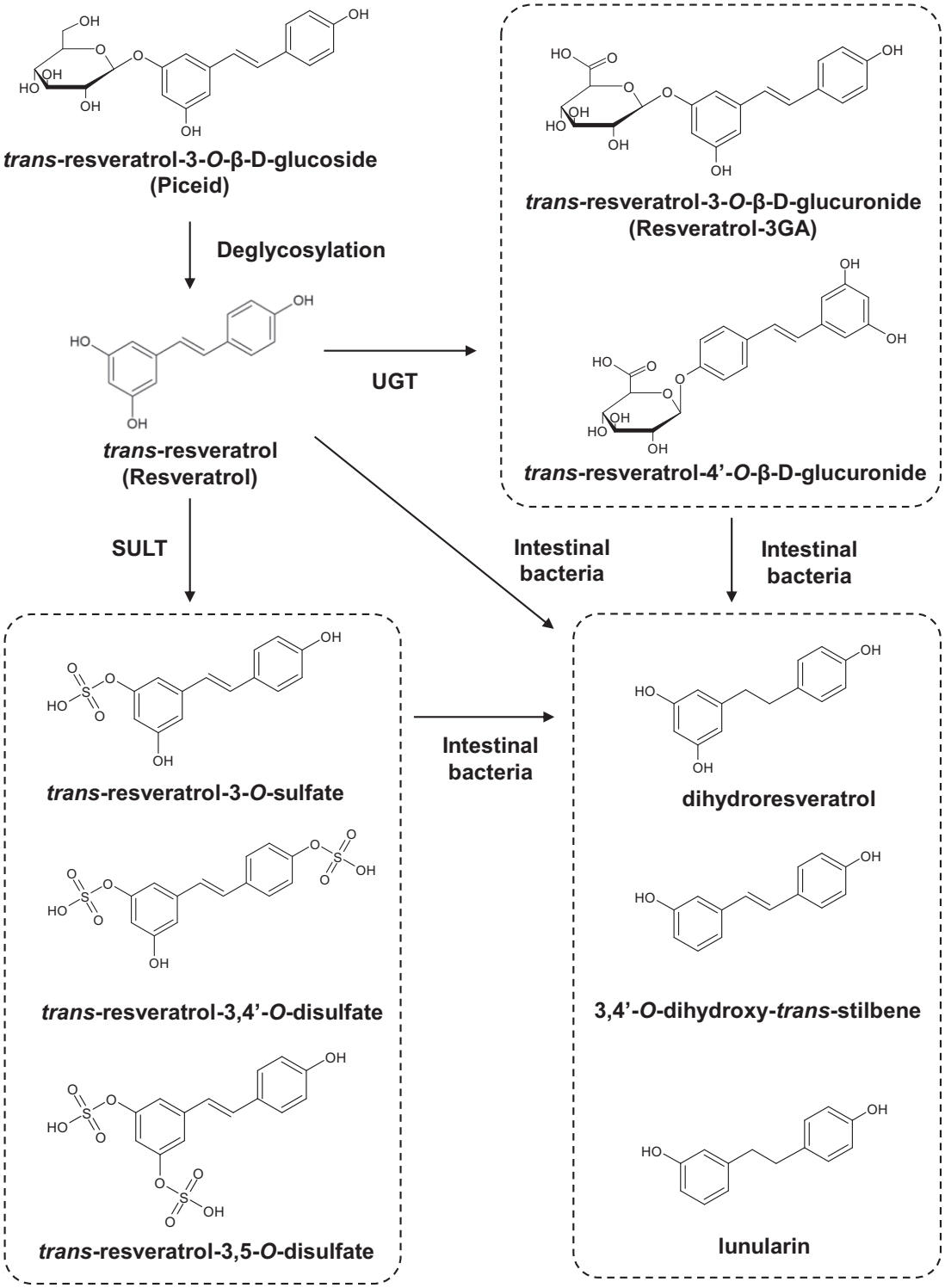

Figure 1. Chemical structures and metabolic pathways of resveratrol and piceid in human.

Jurkat was obtained through the RIKEN BRC under the National Bio-Resource Project of the MEXT/AMED, Japan. Both cell lines were cultured in RPMI1640 supplemented with 10% heat-inactivated FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37 °C and 5% CO_2.

THP-1 cells were differentiated into macrophage-like cells by treating them with 100 ng/mL PMA (162 nM) for 5 days (Chanput, Mes and Wichers 2014). After renewal of the culture media, the cells were incubated for an additional day and subsequently used in the experiments as THP-1 macrophages.

Preparation for murine peritoneal macrophages

BALB/c mice (Japan SLC, Inc., Shizuoka, Japan) were housed under specific pathogen-free conditions with a 12-h light-dark cycle (8:00-20:00) at 25 ± 2 °C and 55 ± 10% relative humidity. The

mice had free access to water and a gamma-irradiation-sterilized medium-fat diet (Oriental Yeast Co., Ltd., Tokyo, Japan). Experiments were conducted in adherence to the ethical guidelines for animal experimentation by the Institute of Health Bioscience, Tokushima University, Japan, and were approved by the institution review board of the animal ethics committee (T2020-107).

Male BALB/c mice (8-12 weeks old) received 2 mL of 3% thioglycolate media into the peritoneal cavity. After 4 days, mice were euthanized with carbon dioxide, and the peritoneal lavage fluid was collected using PBS (-). Peritoneal exudate cells were obtained from the peritoneal lavage fluid, and red blood cells within the cells were lysed using an ammonium chloride solution (0.17 M NH_4Cl, 10 mM Tris-HCl, 0.25 mM EDTA) during a 15-min incubation at room temperature. After washing with medium, the cells were seeded into an appropriate plate. Following a 2-h incubation in a CO_2 incubator, nonadherent cells were washed away with medium, leaving only the cells bound to the plate. These adherent cells were then used in the experiment as murine peritoneal macrophages.

MTT assay

THP-1 macrophages (2×10^4 cells/well) and murine peritoneal macrophages (6×10^4 cells/well), seeded into a 96-well plate, were treated with resveratrol, piceid, or 0.1% dimethyl sulfoxide (DMSO) as a vehicle for 24 h. MTT solution was then added to each well and incubated for 2 h. Formazan crystals generated were completely dissolved with DMSO, and the absorbance of the resulting solution was measured using a Multiskan GO Microplate spectrophotometer (Thermo Fisher Scientific) at a measurement wavelength of 570 nm and a reference wavelength of 650 nm.

Efferocytosis assay

THP-1 macrophages and murine peritoneal macrophages, seeded in a 12-well plate (1×10^6 cells/well), were treated with resveratrol, piceid, or 0.1% DMSO (used as a vehicle) for 24 h. Jurkat cells were treated with 1 μM staurosporine for 3 h to induce apoptosis and then incubated with 1 μM CFSE for 15 min for fluorescent labeling. Macrophages were co-cultured with apoptotic Jurkat cells at a 1:1 ratio for 2 h to induce efferocytosis. Subsequently, macrophages were washed with PBS (-) 3 times to remove unengulfed apoptosis cells, fixed with formalin solution, and observed under a fluorescence microscope. Apoptotic cells completely engulfed by macrophages were counted as engulfed apoptotic cells, and the efferocytic index was calculated as follows: Efferocytic index (%) = Number of engulfed apoptotic cells/Number of macrophages × 100. This index served as a measure for efferocytosis activity of macrophages.

In the experiment investigating the effects of molecules secreted from macrophages on efferocytosis activity, the culture supernatant of macrophages treated with food ingredients for 24 h was transferred to untreated macrophages. Immediately following this, these macrophages were co-cultured with CFSE-labeled apoptotic cells for 2 h. Subsequently, macrophages were washed with PBS (-) 3 times, fixed with a formalin solution, and observed under a fluorescence microscope to calculate the efferocytic index.

Real-time reverse transcription-polymerase chain reaction

THP-1 macrophages were differentiated in a 6-well plate (2×10^6 cells/well) and treated with resveratrol, piceid, or 0.1% DMSO (used as a vehicle) for 24 h. The culture medium was then removed, and the cells were washed with ice-cold PBS (-). Total RNA was isolated using Sepasol®-RNA I Super G according to the manufacturer's instructions. The RNA concentration was measured using a μDrop plate and Multiskan GO Microplate spectrophotometer. After reverse transcription into cDNA with a ReverTra® Ace qPCR RT Master Mix with gDNA Remover according to the manufacturer's instructions, real-time PCR was performed using the Light Cycler Nano System (Roche Diagnostics K.K., Tokyo, Japan) with the THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd.) and gene-specific primers. The primers used in this study are as follows: human MFG-E8, 5′-GCACTCTGCGCTTTGAGCTA-3′(forward) and 5′-TTGTCAGGGATGCTGTTATTCTTC-3′ (reverse); human αV, 5′-ACTGGCTTAAGAGGGCTGTG-3′ (forward) and 5′-TGCCTTACAAAAATCGCTGA-3′ (reverse); human β3, 5′-AGACACTCCCACTTGGCATC-3′(forward) and 5′-TCCTCAGGAAAGGTCCAATG-3′(reverse); human β5, 5′-AGCCTATCTCCACGCACACT-3′ (forward) and 5′-CCTCGGAGAAGGAAACATCA-3′ (reverse); human Gas6, 5′-ATCAAGGTCAACAGGGATGC (forward) and 5′-CTTCTCCGTTCAGCCAGTTC-3′ (reverse); human Axl, 5′-GACCGGCCAAGTTTTACAGA-3′ (forward) and 5′-ATAACCTCCACCCTCATCCA-3′ (reverse); human MerTK, 5′-TGCCCTGGGAATGGAGTATC-3′ (forward) and 5′-ATCTTAGCAATGCGGCCTTG-3′ (reverse); human GAPDH, 5′-CATGAGAAGTATGACAACAGCCT-3′ (forward) and 5′-AGTCCTTCCACGATACCAAAGT-3′ (reverse). The fold changes of the target gene (MFG-E8 and Gas6) expression were calculated using the comparative Ct method (ΔΔCT method) and normalized to the housekeeping gene (GAPDH) expression.

ELISA

ELISA was performed to quantify the secreted protein levels of MFG-E8 and Gas6 in cell culture medium. Macrophages seeded in a 12-well plate (1×10^6 cells/well) were treated with resveratrol, piceid, or 0.1% DMSO (used as a vehicle) for 24 h. The culture supernatants were collected and utilized for ELISA. The Gas6 protein level in the culture supernatant was measured using the Human Gas6 DuoSet ELISA (R&D Systems, Inc., Minneapolis, MN, USA), while the MFG-E8 protein level was measured using the Human MFG-E8 Quantikine ELISA Kit (R&D Systems). Measurements were conducted following the manufacturer's instructions.

Western blot analysis

THP-1 macrophages were differentiated in a 100 mm dish (8×10^6 cells/dish) and treated with resveratrol, piceid, or 0.1% DMSO (used as a vehicle) for 24 h. Whole cell lysates were prepared, and a western blot analysis was performed for MFG-E8, Gas6, and β-actin (loading control) as previously described (Abe-Kanoh *et al.* 2019).

RNA interference

Predesigned siRNAs targeting MFG-E8 (Thermo Fisher Scientific) or nonspecific control siRNAs (Control siRNA-A, Santa Cruz Biotechnology, Inc.) were transfected into the cells following the manufacturer's instructions provided by HiperFect Transfection Reagent (QIAGEN, Venlo, Netherlands). Briefly, THP-1 cells (2.5×10^5 cells/well in a 12-well plate) were treated with 100 ng/mL PMA for 24 h to differentiate into macrophages, and the culture medium was renewed. The siRNA solution, diluted with medium without FBS, was mixed with HiperFect Transfection Reagent and incubated at room temperature for 5-10 min. This mixed solution was then treated to THP-1 macrophages for 6 h at 37 °C, followed by the addition of normal culture medium

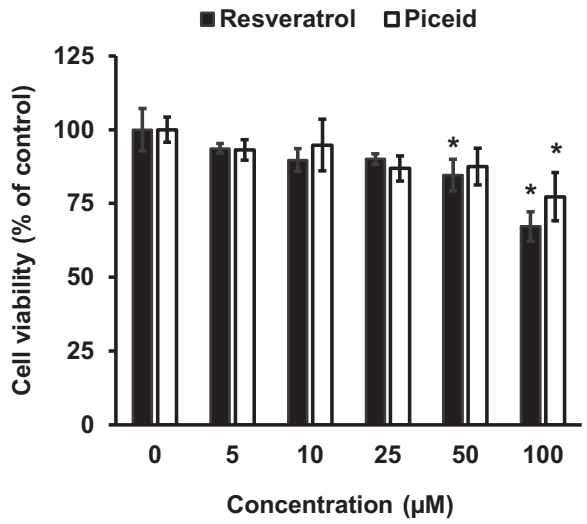

Figure 2. Effects of resveratrol and piceid on cell viability in THP-1 macrophages. THP-1 macrophages were treated with resveratrol or piceid for 24 h and MTT assay was performed to determine cell viability. The values represent the mean ± SD of 3 separate experiments. Data were analyzed by a one-way ANOVA, followed by Dunnett *post hoc* test, using R software, version 4.1.2 (*$P < .05$ vs control).

to the plate. After a 72-h incubation, cells were assayed using the appropriate protocol. It is important to note that the ratio of macrophages to apoptotic cells was 1:10 in the efferocytosis assay performed after the siRNA transfection.

Statistical analysis

All data are presented as mean ± standard deviation. Differences between the means of two groups were assessed using a two-sided Student's *t*-test. For comparisons among the means of 3 or more groups, one-way analysis of variance was employed, followed by Tukey-Kramer or Dunnett post hoc tests. Statistical processing was performed using R software (version 4.1.2), with a significance level set at $P < .05$. Different letters placed above the bars indicate significant differences among treatments for each compound.

Results

Resveratrol and piceid promoted efferocytosis in THP-1 macrophages and murine peritoneal macrophages

Before investigating the effects of resveratrol and piceid on the efferocytic activity in THP-1 macrophages, we first assessed their impact on cell viability to establish a nontoxic concentration range. We observed a significant decrease in cell viability at concentrations of 50 and 100 μM for resveratrol and 100 μM for piceid (Figure 2). Consequently, for the subsequent experiments, cells were treated with resveratrol and piceid concentrations below 25 μM. In the efferocytosis assay, we observed that both 10 and 25 μM concentrations of resveratrol or piceid significantly increased the efferocytic activity compared to the control (Figure 3).

To confirm the contribution of macrophage-secreted molecules to enhanced efferocytosis by resveratrol and piceid, we treated the culture supernatant of THP-1 macrophages, previously treated with 10 μM resveratrol or piceid for 24 h, to untreated THP-1 macrophages. Then, the efferocytic activity was promptly assessed. The culture supernatant of THP-1 macrophages treated with resveratrol or piceid significantly increased the efferocytic activity compared to the control in THP-1 macrophages (Figure 3b and c). Notably, the upregulating effects on the efferocytosis by resveratrol and piceid in THP-1 macrophages were observed from 18 h after treatment (data not shown). Thus, the immediate increase in efferocytosis activity after treatment of the culture supernatant is likely attributed to substances secreted from macrophages treated with resveratrol and piceid, suggesting their involvement in the upregulation of efferocytosis by these compounds in macrophages.

LPS, an outer membrane component of gram-negative bacteria, is known to activate the innate immune system, inducing inflammatory responses in macrophages (Beutler and Rietschel 2003). Treatment with LPS significantly decreased efferocytic activity compared to the control group, whereas this reduction in efferocytosis was counteracted when pretreated with resveratrol. Moreover, pretreatment with piceid showed a tendency to restore the decreased efferocytosis activity induced by LPS treatment, although the result did not reach statistical significance (Figure 3c). These findings suggest the potential preventive effects of resveratrol and piceid on defective efferocytosis under inflammatory conditions.

To confirm whether the promotive effect of resveratrol and piceid on efferocytic activity in human THP-1 macrophages is similarly observed in murine macrophages, we examined the effects of resveratrol and piceid on cell viability and efferocytic activity in murine peritoneal macrophages derived from BALB/c mice. Treatment with resveratrol above 50 μM significantly decreased the cell viability of murine peritoneal macrophages, whereas piceid showed no significant effect at any tested concentration (Figure 4a). When resveratrol or piceid were treated at concentrations below 25 μM, where no cell toxicity was observed, the efferocytic activity significantly increased in the 10 and 25 μM resveratrol and piceid-treated groups compared to the control group (Figure 4b).

Having established the activating effect of resveratrol and piceid on efferocytosis in macrophages, our subsequent investigation focused on evaluating the effect of resveratrol-3GA, a major metabolite of both compounds (Figure 1), on cell viability and efferocytic activity in THP-1 macrophages. While cell viability significantly decreased at concentrations exceeding 50 μM for resveratrol and above 100 μM for piceid (Figure 2), resveratrol-3GA exhibited no effect on cell viability at concentrations up to 100 μM (Figure 5a). Furthermore, resveratrol-3GA showed no effect on efferocytic activity at any tested concentrations (Figure 5b).

Resveratrol and piceid increased the protein secretion of MFG-E8 in THP-1 macrophages

As the culture supernatants of THP-1 macrophages treated with resveratrol and piceid exerted the prompt up-regulating effect of efferocytic activity, we investigated the involvement of opsonins as the molecules contributing to this effect. Opsonins are soluble molecules secreted from macrophages that enhance efferocytic activity by bridging PS on apoptotic cells with receptors on macrophages. In this study, we focused on MFG-E8 and Gas6, the representative opsonins, and examined the effects of resveratrol and piceid on the protein secretion of MFG-E8 and Gas6 in THP-1 macrophages. The secreted protein level of MFG-E8 significantly increased at all tested concentrations of resveratrol and at 10 μM piceid compared to the control (Figure 6a). Although resveratrol exhibited a tendency to increase Gas6 protein secretion compared

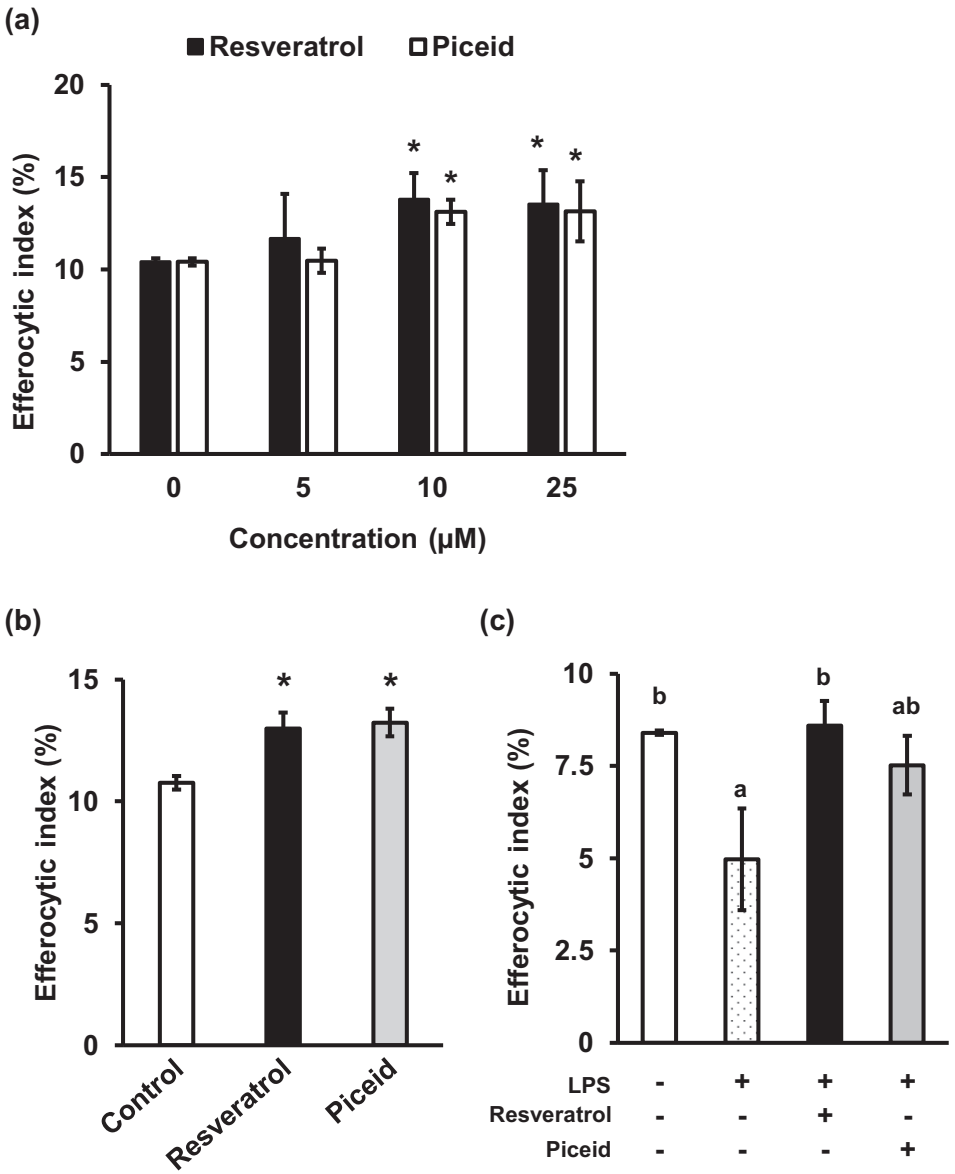

Figure 3. Effects of resveratrol and piceid on efferocytosis in THP-1 macrophages. (a) THP-1 macrophages were treated with resveratrol or piceid for 24 h and the efferocytosis assay was performed. (b) THP-1 macrophages were treated with the culture supernatant of THP-1 macrophages treated with 10 μM resveratrol or 10 μM piceid for 24 h, and the efferocytosis assay was performed. C, THP-1 macrophages were pretreated with 10 μM resveratrol or 10 μM piceid for 1 h and treated with LPS (1 μg/mL) for 23 h. The values represent the mean ± SD of 4 (a) or 3 (b and c) separate experiments. Data were analyzed by a one-way ANOVA, followed by Dunnett (a and b, $^{*}P < .05$ vs control) or Tukey-Kramer *post hoc* test (c), using R software, version 4.1.2. Different letters placed above the bars indicate significant differences among treatments for each compound ($P < .05$).

to the control, neither resveratrol nor piceid at the tested concentrations had a significant effect on the secreted protein level of Gas6 (Figure 6). However, neither resveratrol nor piceid at the concentration that activates efferocytosis increased the intracellular protein levels of MFG-E8 or Gas6 (Figure 6c).

Resveratrol and piceid regulated the gene expression of MFG-E8, Gas6, and their corresponding receptors in THP-1 macrophages

We explored the effect of resveratrol and piceid on the gene expression of MFG-E8 and Gas6 in THP-1 macrophages. Both resveratrol and piceid, at concentrations of 10 and 25 μM, significantly enhanced the gene expression of MFG-E8 compared to the control group (Figure 7). Additionally, 25 μM resveratrol significantly increased the gene expression level of Gas6, while piceid did not exert a significant effect (Figure 7). Considering that opsonin receptors, the membrane-bound proteins, are not secreted from macrophages but play a role in opsonin signaling, we also investigated the effects of resveratrol and piceid on the gene expression of MFG-E8 receptors (integrin αv, β3, and β5) and Gas6 receptors (Axl and MerTK) in THP-1 macrophages. Although, 5-25 μM resveratrol significantly increased the gene expression level of Axl (Figure 7f), both resveratrol and piceid had minimal impact on the gene expression level of other opsonin receptors (Figure 7b-d and g). Collectively, these findings suggest that, in THP-1 macrophages

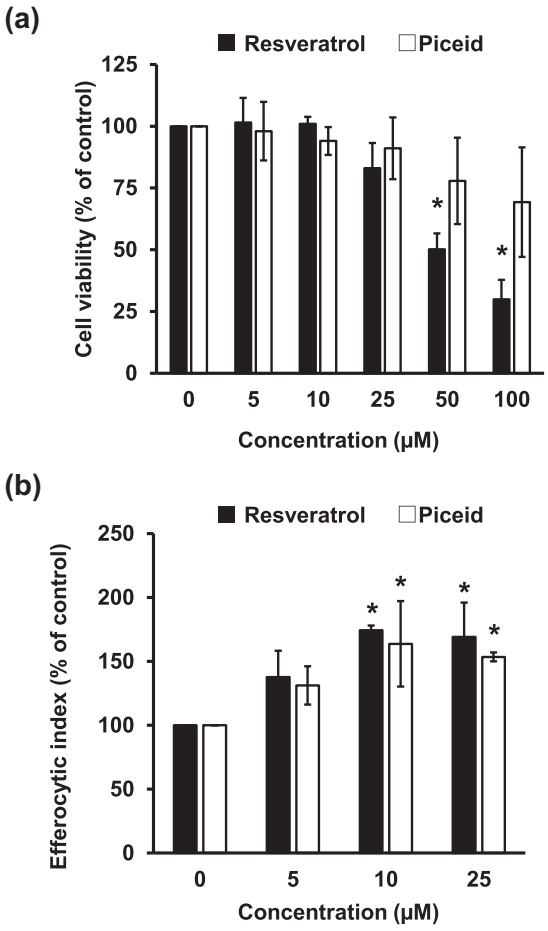

Figure 4. Effects of resveratrol and piceid on cell viability and efferocytosis in murine peritoneal macrophages. Murine peritoneal macrophages were treated with resveratrol or piceid for 24 h and MTT assay (a) or the efferocytosis assay (b) was performed. The values represent the mean ± SD of 3 separate experiments. Data were analyzed by a one-way ANOVA, followed by Dunnett *post hoc* test, using R software, version 4.1.2 ($^*P < .05$ vs control).

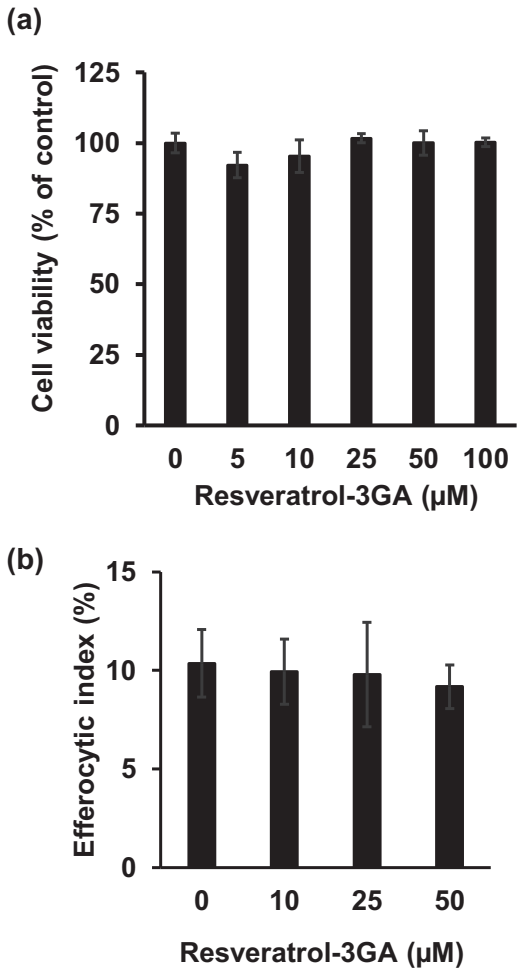

Figure 5. Effects of resveratrol-3GA on cell viability and efferocytosis in THP-1 macrophages. THP-1 macrophages were treated with resveratrol-3GA for 24 h and MTT assay (a) or the efferocytosis assay (b) was performed. The values represent the mean ± SD of 3 separate experiments. Data were analyzed by a one-way ANOVA, followed by Dunnett *post hoc* test, using R software, version 4.1.2 ($^*P < .05$ vs control).

treated with resveratrol and piceid, the increase in MFG-E8 protein excretion is associated with the upregulation of MFG-E8 gene expression.

The enhanced effects of resveratrol and piceid on MFG-E8 protein secretion and efferocytosis in THP-1 macrophages were canceled upon MFG-E8 knockdown

Since resveratrol and piceid significantly promoted efferocytosis and MFG-E8 protein secretion, we investigated the effect of MFG-E8 knockdown on these promoting effects by resveratrol and piceid in THP-1 macrophages. Under control-siRNA transfection, 10 μM resveratrol and piceid significantly increased the protein secretion of MFG-E8, consistent with the results in Figure 6a. The siRNA-mediated knockdown of MFG-E8 decreased the secreted protein level of MFG-E8 by 51% in vehicle (0.1% DMSO)-treated macrophages and completely abolished the promoting effect of resveratrol and piceid on MFG-E8 protein secretion (Figure 8a). Furthermore, under control-siRNA transfection, resveratrol, and piceid significantly enhanced efferocytic activity, consistent with the results in Figure 3a, while MFG-E8 knockdown completely canceled the efferocytic activity enhanced by the treatment of resveratrol and piceid (Figure 8b). These results suggest that resveratrol and piceid promote efferocytosis through the increment of MFG-E8 protein secretion.

Discussion

In this study, we revealed that resveratrol and piceid activate efferocytosis in THP-1 macrophages through the increased protein secretion of the representative opsonin MFG-E8. Additionally, the activation of efferocytosis by resveratrol and piceid was confirmed in experiments using murine peritoneal macrophages (Figure 4b). To further confirm the contribution of MFG-E8 to the efferocytosis-activating effect of resveratrol and piceid, experiments using MFG-E8 knockout macrophages will be necessary in the future. On the other hand, regarding Gas6, another representative opsonin, resveratrol and piceid had little effect on both protein secretion and gene expression levels (Figures 6b and 7e). Since this study assessed the efferocytic activity of macrophages based on their ability to engulf apoptotic cells, future investigations will need to verify whether resveratrol and piceid also promote the process of digesting apoptotic cells within macrophages.

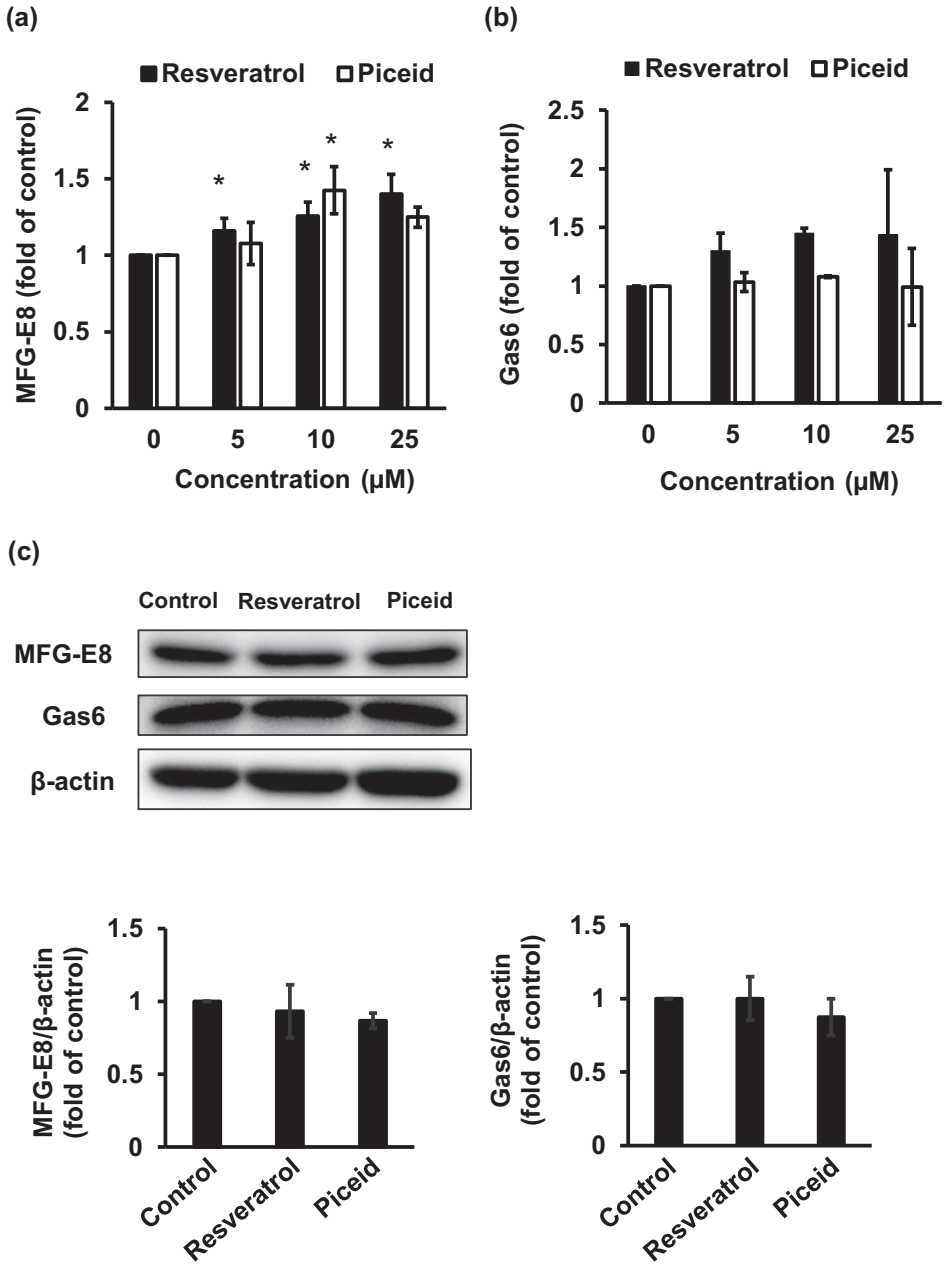

Figure 6. Effects of resveratrol and piceid on the protein secretion and intracellular protein level of MFG-E8 and Gas6 in THP-1 macrophages. THP-1 macrophages were treated with resveratrol or piceid for 24 h and the secreted protein levels of MFG-E8 (a) and Gas6 (b) in the culture supernatant were determined using ELISA. (c) THP-1 macrophages were treated with 10 μM resveratrol or 25 μM piceid for 24 h. The intracellular protein levels of MEG-E8, Gas6, and β-actin (loading control) were determined using a western blot analysis. The values represent the mean ± SD of 3 separate experiments. Data were analyzed by a one-way ANOVA, followed by Dunnett *post hoc* test, using R software, version 4.1.2 ($^*P < .05$ vs control).

In humans, approximately 75% of resveratrol absorbed from the intestine is quickly metabolized, mostly forming glucuronide or sulfate conjugates (Goldberg, Yan and Soleas 2003; Walle *et al.* 2004; Walle 2011). Therefore, oral intake of resveratrol is considered to have low biological availability. In this study, resveratrol-3GA, the glucuronide conjugate of resveratrol, and the main metabolites of both resveratrol and piceid showed no effect on the efferocytic activity of THP-1 macrophages at all tested concentrations (Figure 5b). This suggests that the efferocytosis-activating effect of resveratrol may not occur upon administration or ingestion and may be specific to intestinal macrophages that have direct contact with resveratrol. However, quercetin, another polyphenol, undergoes glucuronide conjugation but is reported to undergo deconjugation by macrophages in an inflammatory environment, exerting its effects in the aglycone form (Ishisaka *et al.* 2013). Furthermore, human vascular endothelial cells have been shown to deconjugate resveratrol-3GA to resveratrol in a concentration-dependent manner (Fernández-Castillejo *et al.* 2019). Co-administration of piperine, a component of black pepper, with resveratrol increases the maximum resveratrol peak concentration in mouse plasma to about 15 times that of the group receiving resveratrol alone (Johnson *et al.* 2011). These results suggest the possibility that resveratrol-3GA, as a metabolite of resveratrol, may undergo deconjugation to resveratrol in an

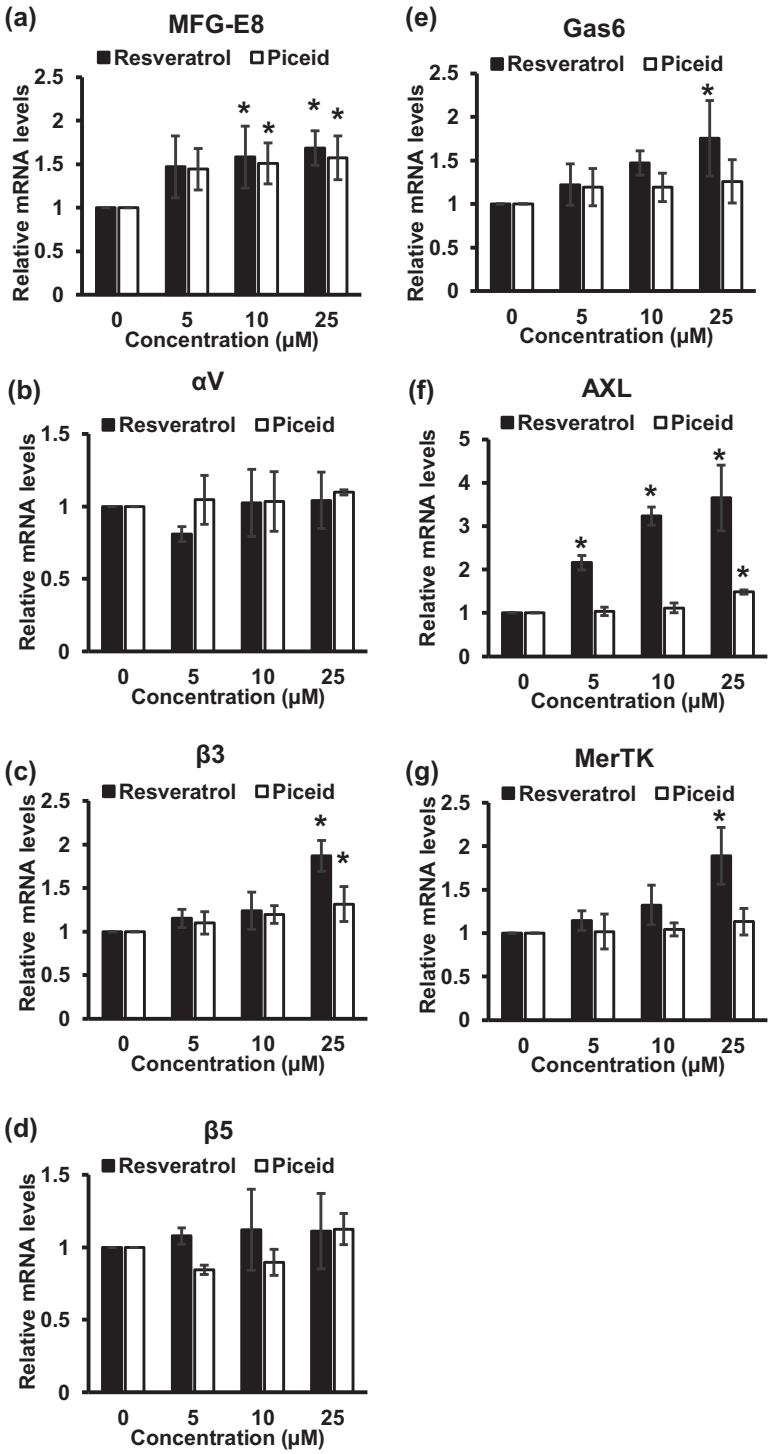

Figure 7. Effects of resveratrol and piceid on the gene expression of MFG-E8, Gas6 and their receptors in THP-1 macrophages. THP-1 macrophages were treated with resveratrol or piceid for 24 h and the relative mRNA levels of MFG-E8 (a), αV (b), β3 (c), β5 (d), Gas6 (e), Axl (f), and MerTK (g) normalized to GAPDH were determined by a real-time RT-PCR. The values represent the mean ± SD of three separate experiments. Data were analyzed by a one-way ANOVA, followed by Dunnett *post hoc* test, using R software, version 4.1.2 ($^*P < .05$ vs control).

inflammatory environment and exert its effects on macrophages. Alternatively, a combination of ingested components may efficiently affect macrophages. Resveratrol accumulates in THP-1 macrophages, and piceid accumulates in macrophages in both piceid and resveratrol form, with the accumulation being less compared to resveratrol treatment (data not shown). This indicates the possibility that resveratrol, when taken up by macrophages, may enhance efferocytosis. Therefore, further study is required to examine whether resveratrol-3GA is deconjugated to resveratrol and accumulates in macrophages in resveratrol form under inflammatory conditions, thus improving efferocytosis.

Macrophages dynamically respond to the tissue environment by altering their properties. It is known that macrophage phenotypes can reversibly change in response to extracellular

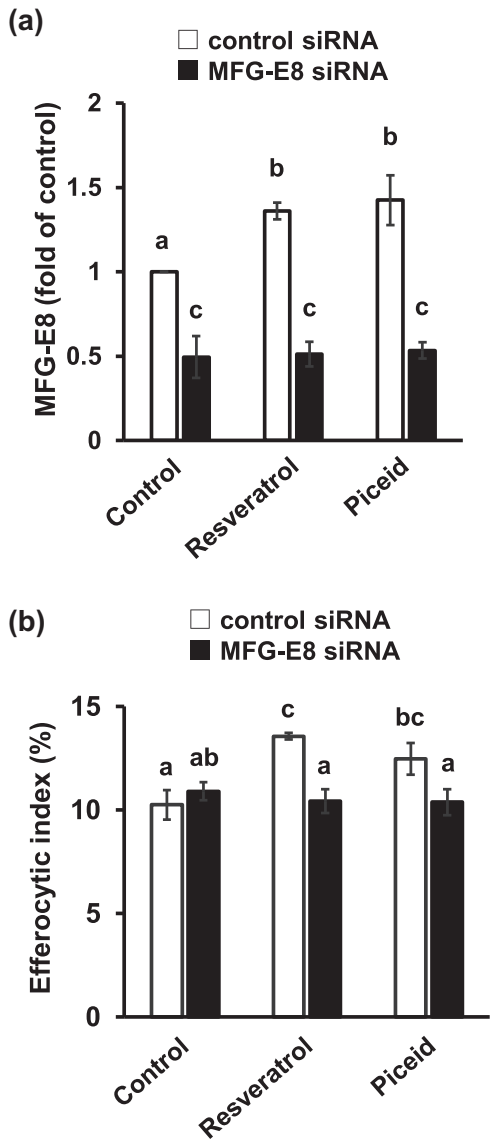

Figure 8. Effects of MFG-E8 knockdown on the enhanced efferocytosis and increased in MFG-E8 excretion induced by the treatment of resveratrol or piceid in THP-1 macrophages. THP-1 macrophages were transfected with control siRNA or MFG-E8 siRNA and treated with 10 μM resveratrol or 10 μM piceid for 24 h. After that, the concentration of MFG-E8 in the culture supernatant was determined by ELISA (a) and the efferocytosis assay was performed (b). The values represent the mean ± SD of three separate experiments. Data were analyzed by a one-way ANOVA, followed by Tukey-Kramer *post hoc* test, using R software, version 4.1.2. Different letters placed above the bars indicate significant differences among treatments for each compound ($P < .05$).

signals, including various cytokines and LPS. Stimulation can lead to polarization toward the proinflammatory M1 or the anti-inflammatory M2 phenotype, depending on the presence or absence of specific signals like interferon-gamma or LPS. M1 macrophages typically release inflammatory cytokines such as IL-6 and IL-1β, and inducible nitric oxide synthase, primarily acting in host defense, but with reported decreased efferocytosis activity (McPhillips *et al.* 2007; Benoit, Desnues and Mege 2008). Although this study did not measure the polarization marker of macrophages after pretreatment with resveratrol or piceid during LPS stimulation, we confirmed that powerful proinflammatory agents LPS resulted in a decrease in efferocytosis activity, as previously reported (Michlewska *et al.* 2009). Furthermore, we revealed that pretreatment with resveratrol prevented the decrease in efferocytic activity caused by LPS (Figure 3c). These results suggest the possibility that resveratrol can contribute to alleviating the inflammatory state by preventing the decrease in efferocytosis activity in macrophages under inflammatory conditions. Resveratrol is known to exert anti-inflammatory effects by inhibiting the NF-κB pathway and reducing inflammatory cytokine production *via* SIRT1 activation (Kauppinen *et al.* 2013; Farkhondeh *et al.* 2020). However, it is yet to be clarified whether the efferocytosis-activating effect of resveratrol contributes to the improvement of chronic inflammatory diseases. Therefore, further verification through *in vivo* experiments using disease models, such as reporting the inhibitory effect of resveratrol on atherosclerotic progression in ApoE-deficient mice (Li *et al.* 2019; Zhou *et al.* 2020), will be necessary.

Resveratrol has gained attention as an activator of *SIRT1*, a longevity gene, leading to increased research on diseases associated with both SIRT1 and resveratrol. SIRT1 has been reported to regulate the expression of various signal molecules, including transcription factors, and plays a crucial role in metabolic control within the body (Haigis and Sinclair 2010; Iside *et al.* 2020). In addition to resveratrol, piceid has also been reported to have anti-inflammatory and antioxidant effects through SIRT1 activation (Xie *et al.* 2012; Huang *et al.* 2015). STAT6, a molecule activated by SIRT1, is a major intracellular signaling molecule of the IL-4/IL-4 receptor signaling. Upon phosphorylation, STAT6 forms dimers and translocates to the nucleus, primarily activating the transcription of genes specific to Th2-type immune responses. Activation of STAT6 has been reported to increase the secretion of Gas6 by alveolar macrophages and activate efferocytosis (Nepal *et al.* 2019). SIRT1 is also known to activate PPARγ, a member of the nuclear receptor superfamily, which induces peroxisome proliferation and regulates the expression of gene clusters closely related to cell metabolism and differentiation. Activation of the PPARγ pathway results in increased expression of MFG-E8, Gas6, and CD36 and enhanced efferocytosis (Luo *et al.* 2016). Therefore, future studies aimed at elucidating the involvement of SIRT1 signaling in resveratrol-promoted efferocytosis will be essential.

In summary, this study revealed that resveratrol and piceid enhance efferocytosis in human-derived THP-1 macrophages by increasing the protein secretion of the opsonin MFG-E8. Additionally, resveratrol was shown to reverse the reduction in efferocytic activity caused by LPS, suggesting the potential of resveratrol to mitigate inflammation through efferocytosis activation. The findings gained from this research are expected not only to provide scientific evidence for the functional properties of dietary components but also to contribute to the development of functional foods based on novel molecular mechanisms and the establishment of prevention and therapeutic strategies for chronic inflammatory diseases.

Data availability

Data available on request. The data underlying this article will be shared on reasonable request to the corresponding author.

Author contribution

N.A.-K. designed the experiments. J.W., Y.H., M.H.-K., A.N., and N.A.-K. conducted the experiments and analyzed the data. T.S. and W.Y. assisted with the experiments and contributed to the discussions. Y.H. and N.A.-K. interpreted the experiments and wrote the manuscript.

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Disclosure statement

The authors declare no conflict of interest.

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