Published in final edited form as:

Arch Oral Biol. 2019 January ; 97: 116–121. doi:10.1016/j.archoralbio.2018.10.020.

Resveratrol represses tumor necrosis factor α /c-Jun N-terminal kinase signaling via autophagy in human dental pulp stem cells

Feng-Ming Wang^{a,*}, Zhiai Hu^b, Xiaohua Liu^b, Jian Q. Feng^b, Robert. A. Augsburger^a, James L. Gutmann^c, Gerald N. Glickman^a

^aDepartment of Endodontics, Texas A&M College of Dentistry, Dallas, TX, USA

^bDepartment of Biomedical Sciences, Texas A&M College of Dentistry, Dallas, TX, USA

^cProfessor Emeritus, Texas A&M College of Dentistry, Dallas, TX, USA

Abstract

Objectives: To study the effects of polyphenol resveratrol on TNF α -induced inflammatory signaling as well as the underlying mechanism in human dental pulp stem cells (DPSCs).

Materials and methods: Human DPSCs were cultured and treated by TNF α in the presence or absence of resveratrol. NF- κ B and mitogen-activated protein kinase (MAPK) signaling pathways were analyzed by Western blotting and immunofluorescence staining. *Interleukin 6 (IL6)* and *interleukin 8 (IL8)* mRNA levels were analyzed by reverse transcription polymerase chain reaction. For the mechanistic study, autophagy was examined and further manipulated by gene silencing of *Atg5* using siRNAs. Statistical analysis was performed by Student's *t*-test, and values of $p < 0.05$ were considered significant.

Results: Upon TNF α treatments, neither degradation of I κ B α nor the phosphorylation and nuclear translocation of p65 NF- κ B were inhibited by resveratrol at different concentrations. In contrast, resveratrol dramatically inhibited TNF α -induced phosphorylation of c-Jun N-terminal kinase (JNK) MAPK. Furthermore, resveratrol activated autophagy, as evidenced by the accumulated autophagic puncta formed by lipid bound LC3B in resveratrol-treated cells. Intriguingly, both resveratrol and JNK inhibitor SP600125 suppressed TNF α -induced *IL6* and *IL8* mRNA expression ($P < 0.05$). Silencing autophagy gene *Atg5* led to the hyper-activation of JNK and augmented TNF α -induced *IL6* and *IL8* mRNA expression ($P < 0.05$).

Conclusions: The results suggest that resveratrol suppresses TNF α -induced inflammatory cytokines expressed by DPSCs through regulating the inhibitory autophagy-JNK signaling cascade. Resveratrol might be beneficial to ameliorate pulpal damage during the acute phase of inflammation in vital pulp therapy.

Keywords

Autophagy; Dental pulp stem cells; Inflammation; Resveratrol; TNF α .

*Corresponding author at: Department of Endodontics, Texas A&M College of Dentistry, 3302 Gaston Ave, Dallas, TX 75246, USA. fengmingwang@tamhsc.edu (F.-M. Wang).

Conflict of interest

The authors deny any conflicts of interest related to this study.

1. Introduction

One objective within the scope of endodontics is to preserve and maintain healthy vital pulp tissue, which allows the normal functions of the dental pulp. However, even though infectious agents are removed during vital pulp therapy, inflammation induced by noninfectious endogenous agents can be detrimental which, if in excess, can lead to tissue damage. Due to an incomplete understanding of the interplay between inflammation and regeneration (Smith, Smith, Shelton, & Cooper, 2012) and the uncertain prognosis of vital pulp therapy (Bjorndal et al., 2017; Mente et al., 2010), pulpal inflammation remains a standard indicator for root canal treatment that involves devitalization of the tooth. A thorough understanding of the inflammatory response in dental pulp injury would be beneficial to improve vital pulp therapy procedures.

Tumor necrosis factor α (TNF α) is one of the pivotal cytokines that initiates the innate inflammatory response in the pulp. It is produced chiefly by macrophages populated locally following pulp tissue injury or infection. During irreversible pulpitis, TNF α gene expression was found to be significantly up-regulated (Kokkas, Goulas, Varsamidis, Mirtsou, & Tziafas, 2007). TNF α in turn activates multiple signaling cascades to induce the expression of additional cytokines that stimulate the acute-phase of the inflammatory response and attract neutrophils to the injury site. A recent animal study demonstrated that conditional over-expression of TNF α in pulp tissue alone could produce inflammation similar to pulpitis (Hall et al., 2016). Chang and colleagues (Chang, Zhang, Tani-Ishii, Shi, & Wang, 2005) demonstrated that TNF α activates the Nuclear factor- κ B (NF- κ B) signaling pathway while increasing the mRNA expression of *Interleukin 6* (IL6) and *Interleukin 8* (IL8) in human dental pulp stem cells (DPSCs). In fact, in addition to the NF- κ B signaling pathway, c-Jun N-terminal Kinase (JNK) also transduces TNF α signaling (Toubal, Treuter, Clement, & Venteclef, 2013). Activation of and cross-talk between Activator Protein-1 (AP-1), the substrate of JNK, and NF- κ B are reported to contribute to transcription of IL6 and IL8 (Roebuck, 1999; Vanden Berghe, De Bosscher, Boone, Plaisance, & Haegeman, 1999). In renal epithelial cells, JNK inhibitor was found to repress TNF α -induced expression of IL6 (de Haij et al., 2005). However, it remains to be shown how NF- κ B or JNK signaling pathway mediates TNF α -induced expression of IL6 and IL8 in pulpal inflammation.

Resveratrol (trans-3, 4', 5-trihydroxystilbene) is a polyphenol that can be found abundantly in red wine and is produced by plants in response to external attack. The fact that resveratrol mediates the cardioprotective effects of red wine has triggered increasing research interest in this compound. Resveratrol can modulate a variety of biological processes, such as DNA repair, apoptosis, metabolism, and inflammation, by mimicking caloric restriction to extend health and longevity (Baur & Sinclair, 2006; Yuan & Marmorstein, 2013). As part of the mechanism, resveratrol induces the cyto-protective self-digestive process - autophagy (Rubinsztein, Marino, & Kroemer, 2011). The protective effects of resveratrol in inflammation have been demonstrated in lipopolysaccharide (LPS)-induced airway inflammation (Birrell et al., 2005), inflammatory bowel disease (Nunes, Danesi, Del Rio, & Silva, 2017), and osteoarthritis (Nguyen, Savouret, Widerak, Corvol, & Rannou, 2017). Decreased production of pro-inflammatory factors and subsequent modulation of cellular

and humoral immune responses, mainly mediate resveratrol's effect in inflammation. Lee and colleagues (2011) (Lee et al., 2011) showed that resveratrol attenuated LPS-induced *IL8* mRNA in human dental pulp cells. Nevertheless, it is largely unknown whether or not resveratrol could modulate pulpal inflammation.

Therefore, the purpose of this study was to determine whether resveratrol affected TNF α signaling and TNF α -induced expression of IL6 and IL8 in human DPSCs. Furthermore, the mechanisms by which resveratrol regulates TNF α signaling were investigated as well.

2. Materials and methods

2.1. Reagents and chemicals

Minimum Essential Medium (MEM)- α without ascorbic acid (A10490), Gibco™Fetal Bovine Serum (FBS, A3160401), OPTI-MEM I (31985–070), Lipofectamine RNAiMAX transfection reagent (A13778100), ECL Western Blotting substrate (#32106), Alexa Fluor® 488 goat anti-rabbit IgG (H + L) conjugate (A-11008), Alexa Fluor® 633 phalloidin (A22284), and ProLong™Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (P36931) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Antibiotic-antimycotic solution (Catalog # 30–004-CI) was procured from Cellgro (Herndon, VA, USA). Dimethyl sulfoxide (DMSO), resveratrol (R5010) and c-Jun N-terminal kinase (JNK) inhibitor SP600125 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anti-LC3B antibody (#3868), anti-phos-NF- κ B p65 (Ser536) antibody (#3033), anti-NF- κ B p65 antibody (#8242), anti-I κ B α antibody (#4814), anti-phos-p38 (Thr180/Tyr182) antibody (#4511), anti-p38 antibody (#8690), anti-Stress-Activated Protein Kinase (SAPK)/JNK antibody (#9258), anti-phos-SAPK/JNK (T183/Y185) antibody (#4668), anti-ATG5 antibody (#2630), and horseradish peroxidase-conjugated secondary antibodies (#7074 and #7076) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti- β -actin antibody (sc-47778) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human TNF α was purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Cell culture

Human DPSCs were provided by Dr. Songtao Shi (University of Pennsylvania School of Dental Medicine, USA) (Gronthos, Mankani, Brahimi, Robey, & Shi, 2000). Cells were cultured in MEM- α supplemented with 10% FBS and 1 $\times$ antibiotic-antimycotic solution in a humidified incubator with 5% CO₂ at 37 °C. DPSCs of passages 3–6 were used in the following experiments. When indicated, TNF α was used for treatments at the concentration of 2.5 ng/mL.

2.3. RNA interference

SignalSilence® *Atg5* siRNA I (#6345) and control (#6568) were purchased from Cell Signaling Technology (Beverly, MA, USA). siRNAs were introduced into DPSCs with Lipofectamine RNAiMAX transfection reagent according to the manufacturer's instructions. In brief, cells were plated (30,000 per well of 12-well plate) in 800 μ L antibiotics-free medium and incubated overnight. Transfection media (200 μ L OPTI-MEM I) containing 20

nM siRNAs and 1 μ L Lipofectamine reagent was added into each well. Cells were then incubated with the siRNA for 24 h prior to analysis or further treatment.

2.4. Western blotting

Cells were lysed in cell lysis buffer following washing with cold phosphate-buffered saline (Wang, Galson, Roodman, & Ouyang, 2011). Protein was extracted after centrifugation at $14,000\times g$ for 15 min at 4 °C. Samples were mixed with $4\times$ Laemmli Sample Buffer and denatured for 5 min at 95 °C. Equal amounts of protein samples were loaded onto a sodium dodecyl sulfate polyacrylamide gel. The proteins blotted on polyvinylidene difluoride membranes were analyzed by using the following primary antibodies: anti-I κ B α (1:1000), anti-phos-p65 (1:1000), anti-p65 (1:1000), anti-phos-p38 (1:1000), anti-p38 (1:1000), anti-phos-JNK (1:1000), anti-JNK (1:1000), anti-LC3B (1:1000), anti-ATG5 (1:1000), and anti- β -actin (1:2000). Signals were detected using horseradish peroxidase-conjugated secondary antibodies (1:2000) and ECL reagents.

2.5. Immunofluorescence staining

Cells grown on chamber-slides were treated as indicated. Following washing with cold phosphate-buffered saline, cells were fixed in 4% phosphate-buffered paraformaldehyde (pH 7.2) for 15 min at 4 °C. Permeabilized cells were stained using anti-NF- κ B p65 antibody or anti-LC3B antibody as the primary antibody and Alexa Fluor® 488 goat anti-rabbit IgG (H + L) conjugate as the secondary antibody. Cells were further counter-stained with Alexa Fluor® 633 Phalloidin for 45 min at 37 °C and mounted with ProLong Gold Antifade Mountant with DAPI. Staining of p65 or autophagy puncta (Green), Filamentous actin (Phalloidin, red), and nucleus (blue) was visualized under a confocal laser microscope (TCS SP5, Leica, Buffalo, USA).

2.6. Qualitative reverse transcription polymerase chain reaction

Total RNA was isolated using Trizol® reagent according to manufacturer's instruction. Reverse transcription was made using a GoScript™ Reverse Transcription System kit (Promega, Madison, WI). An aliquot of the complementary DNA product was subjected to PCR using SsoAdvanced SYBR Green Supermix and CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA). PCR condition consisted of an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. 18S ribosomal RNA was applied as an internal control for data analysis. The nucleotide sequences of primers used for PCR are as follow: *IL6* 5'-GCCAGCTATGAACTCCTTCT-3' and 5'-GAAGGCAGCAGGCAACAC-3'; *IL8* 5'-GGCACAACTTTCAGAGA CAG-3' and 5'-ACACAGAGCTGCAGAAATCAGG-3'; 18S ribosomal RNA 5'-ATCCCTGAAAAGTTCCAGCA-3' and 5'-CCCTCTTGGTGAGGTCA ATG-3'.

2.7. Statistical analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical significance was analyzed by Student's *t*-test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effect of resveratrol on TNF α signaling in DPSCs

Initially, the signaling responses by TNF α treatment in the presence or absence of resveratrol were examined, addressing both NF- κ B and MAPK pathways. TNF α in a time course manner induced a dramatic degradation of I κ B α , the inhibitor of NF- κ B pathway as shown in Fig. 1A. Although resveratrol obviously delayed the degradation of I κ B α at the 5-minute post-treatment time point and rebound of I κ B α at the 60-minute post-treatment time point, resveratrol did not appear to abrogate this process. Strikingly, the phosphorylation of p65 by TNF α treatment was not influenced by the presence of resveratrol. The nuclear translocation of p65 was obvious in the cells treated by TNF α . However, this process was not hindered by resveratrol treatment (Fig. 1B). In addition, none of the treatments affected DPSCs vitality according to the blue-fluorescent DNA staining that showed no sign of apoptosis (Fig. 1B).

In the same treatments, phosphorylation of p38 MAPK was not affected by the presence of resveratrol. Surprisingly, phosphorylation of JNK MAPK was apparently reduced by the presence of resveratrol in a dose-dependent manner (Fig. 1A).

3.2. Both resveratrol and JNK inhibitor suppressed TNF α -induced IL6 and IL8 mRNA expression in DPSCs

We next examined whether resveratrol affected the regulation of the expression of inflammatory genes, such as *IL6* and *IL8*, when cells were exposed to TNF α . With no exception, TNF α strongly induced the expression of *IL6* and *IL8* mRNA (Fig. 2). Interestingly, pretreatment of cells with resveratrol significantly inhibited the induction of *IL6* (Fig. 2A) and *IL8* (Fig. 2B) mRNA in cells stimulated by TNF α . Further experiments using JNK inhibitor SP600125 showed that blockage of JNK signaling pathway resulted in the reduction of TNF α -induced *IL6* (Fig. 2C) and *IL8* (Fig. 2D) mRNA expression, similarly as did resveratrol.

3.3. Resveratrol activated autophagy in DPSCs

Since the TNF α -JNK signaling was interrupted by the pretreatment of resveratrol, we questioned whether resveratrol would affect autophagy, a potential negative regulator of JNK activation (Tang et al., 2013). Subsequently, the same experimental samples in Fig. 1a were analyzed for the activation of autophagy. Resveratrol apparently increased the levels of membrane bound form of LC3B (LC3B-II), an indicator of autophagosome formation for the whole period of TNF α exposure, as shown in Fig. 3A. Confocal microscopy further demonstrated that resveratrol treatment increased autophagic puncta in the cytoplasm compartment of the DPSCs (Fig. 3B). These observations suggested that activation of autophagy by resveratrol might be associated with the down-regulation of TNF α -JNK signaling.

3.4. Modulation of autophagy by Atg5 siRNA augmented TNF α -induced IL6 and IL8 mRNA expression in DPSCs

Using SignalSilence® siRNAs, we reduced the expression of endogenous ATG5 protein in DPSCs (Fig. 3C). Since the ATG5 is critical for autophagosome formation (Matsuzawa-Ishimoto, Hwang, & Cadwell, 2018), the membrane bound form of LC3B (LC3B-II) was reduced significantly at the basal condition in cells transfected with *Atg5* siRNA. Surprisingly, the phosphorylation of JNK was increased simultaneously. This observation matched with the aforementioned finding that the increased LC3B-II was accompanied by the decreased phosphorylation of JNK (Figs. 1A, 3 A).

Lastly, we examined the expression of *IL6* and *IL8* mRNA in DPSCs which were transfected with *Atg5* siRNA or its control. It was found that the TNF α -induced *IL6* and *IL8* mRNA expression was strongly augmented in cells transfected with *Atg5* siRNA (Fig. 3D, E), indicating that the impairment of autophagy enhanced inflammatory cytokine production in TNF α -treated DPSCs.

4. Discussion

This study initially demonstrated that JNK signaling pathway was involved in TNF α signaling to regulate the production of pro-inflammatory cytokines such as IL6 and IL8 in human DPSCs. Interestingly, targeting JNK signaling by its specific inhibitor (SP600125) or resveratrol, significantly attenuated TNF α -induced expression of *IL6* and *IL8* mRNA. On one hand, IL6 is a pleiotropic cytokine that plays an active role in immune responses and the development of the acute phase response (Heinrich, Castell, & Andus, 1990). On the other hand, IL8 is a chemokine that functions primarily to promote the recruitment and activation of neutrophils to the sites of acute inflammation, where neutrophils not only kill bacteria by phagocytosis but also destroy affected tissue by secreting proteases and generating oxidative oxygen species (Kobayashi, 2008). It is worth noting that targeting JNK signaling pathway has been shown to reduce TNF α -enhanced expression of matrix metalloproteinase (MMP)-3, a key matrix metalloproteinase in inflammatory tissue damage, in human dental pulp cells (Goda et al., 2015). Given the important roles of regulating pro-inflammatory cytokines, JNK signaling pathway appears to be a promising target for therapeutics in the reduction of pulpal inflammation in addition to NF κ B signaling pathway. Furthermore, TNF α had been shown previously to promote an odontoblastic phenotype in cultured human dental pulp cells through p38 MAPK pathway (Paula-Silva, Ghosh, Silva, & Kapila, 2009). In our study, resveratrol did not affect the TNF α -induced phosphorylation of p38, suggesting that resveratrol might be able to maintain the induction potential of TNF α for odontoblastic differentiation while suppressing JNK signaling pathway. Although it is commonly believed that p65 NF- κ B is a master transcription factor in the regulation of inflammatory response to TNF α (Baldwin, 2001), in this study resveratrol did not affect the phosphorylation or nuclear translocation of p65 NF- κ B, while it delayed the degradation of I κ B. Further investigations are required to determine whether the delayed degradation of I κ B plays a role in the suppression of TNF α -induced expression of inflammatory cytokines.

The present data suggest that resveratrol inhibited the phosphorylation of JNK induced by TNF α in human DPSCs. Similarly, resveratrol has been shown to inhibit phorbol myristate

acetate-induced JNK activation and to regulate tumor growth and invasiveness (Woo et al., 2004). The underlying mechanisms for these outcomes are poorly understood. It is interesting to observe that the activation of the autophagy process by resveratrol was correlated with reduced JNK phosphorylation, while the deactivation of autophagy process by gene silencing of *Atg5* was correlated with enhanced phosphorylation of JNK. These results are consistent with a previous finding that the depletion of *Atg5* in MCF7 cells led to prolonged phosphorylation of JNK during hydrogen peroxide induced oxidative stress (Tang et al., 2013). It appears plausible that autophagy negatively regulated JNK phosphorylation. This regulatory mechanism might provide a previously unexplored role of autophagy in JNK-related human inflammatory diseases. For example, JNK plays a central role in obesity and insulin resistance (Hirosumi et al., 2002). One may speculate that resveratrol's reported therapeutic potential in human obesity and type 2 diabetes (Timmers, Hesselink, & Schrauwen, 2013) could be attributed to the inhibition of JNK activity by the activation of autophagy. Future studies are warranted to identify the molecular mechanisms involved in the regulatory effect of autophagy on JNK signaling pathway.

Although not surprising, to the best of our knowledge, this is the first study demonstrating that resveratrol triggered the autophagic cascade in human DPSCs. Resveratrol treatment mimics caloric restriction and it can (directly or indirectly) activate the deacetylase SIRT1 to stimulate the deacetylation of cytosolic autophagy-relevant proteins (such as ATG5 and LC3) to induce autophagic cascade (Baur & Sinclair, 2006; Morselli et al., 2011). Recently, it was demonstrated that resveratrol induced autophagy by directly inhibiting the mTOR-ULK1 pathway in human cells (Park et al., 2016). Moreover, previous studies have suggested that autophagy functions positively in odontoblastic differentiation (Takanche, Kim, Kim, Han, & Yi, 2018) and that autophagy maintains cellular homeostasis by improving pulpal mitochondrial activity (Y. H. Lee et al., 2015), while the present work further establishes a regulatory loop among resveratrol, autophagy, and the JNK signaling pathway in human DPSCs during TNF α -stimulated inflammatory responses.

Autophagy has been reported to control inflammation by repressing inflammasomes that proteolytically process pro-inflammatory cytokines including interleukin 1 β (IL1 β) to promote their maturation (Lamkanfi & Dixit, 2014). In addition to this post-translational regulation, our study demonstrated that autophagy can also regulate pro-inflammatory cytokines at the transcriptional level. Modulation of autophagy by using *Atg5* siRNA resulted an augmented increase in TNF α -induced expression of *IL6* and *IL8* mRNA in DPSCs. These results echo the findings that inhibition of autophagy augmented LPS-induced expression of *IL6* and *IL8* in human bronchial epithelial cells, resulting in attenuated airway inflammation in response to LPS exposure (Hu et al., 2016). However, the expression of mature IL1 β by human DPSCs was not measured in this study. The regulatory role of autophagy in inflammasomes in DPSCs remains to be further studied.

Taken together, this study demonstrated that resveratrol suppresses TNF α -induced inflammatory cytokines produced by human DPSCs through regulating the inhibitory autophagy-JNK signaling cascade (Fig. 4). It is hypothesized that resveratrol might be beneficial to ameliorate pulpal damage during the acute phase of inflammation in vital pulp

therapy. Further investigations, especially *in vivo* studies, will be necessary to test this hypothesis before resveratrol can be considered for its therapeutic use clinically.

Acknowledgements

The authors would like to thank Dr. Songtao Shi (University of Pennsylvania School of Dental Medicine, USA) for kindly providing the human dental pulp stem cells. This work was supported by the Texas A&M College of Dentistry Research Start-up Fund and the National Institutes of Health [R01DE024979].

References

- Baldwin AS Jr. (2001). Series introduction: The transcription factor NF-kappaB and human disease. *The Journal of Clinical Investigation*, 107(1), 3–6. [PubMed: 11134170]
- Baur JA, & Sinclair DA (2006). Therapeutic potential of resveratrol: The *in vivo* evidence. *Nature Reviews Drug Discovery*, 5(6), 493–506. [PubMed: 16732220]
- Birrell MA, McCluskie K, Wong S, Donnelly LE, Barnes PJ, & Belvisi MG (2005). Resveratrol, an extract of red wine, inhibits lipopolysaccharide induced airway neutrophilia and inflammatory mediators through an NF-kappaB-independent mechanism. *The FASEB Journal*, 19(7), 840–841. [PubMed: 15734790]
- Bjorndal L, Fransson H, Bruun G, Markvart M, Kjaeldgaard M, Nasman P, et al. (2017). Randomized clinical trials on deep carious lesions: 5-Year follow-up. *Journal of Dental Research*, 96(7), 747–753. [PubMed: 28410008]
- Chang J, Zhang C, Tani-Ishii N, Shi S, & Wang CY (2005). NF-kappaB activation in human dental pulp stem cells by TNF and LPS. *Journal of Dental Research*, 84(11), 994–998. [PubMed: 16246929]
- de Haij S, Bakker AC, van der Geest RN, Haegeman G, Vanden Berghe W, Aarbiou J, et al. (2005). NF-kappaB mediated IL-6 production by renal epithelial cells is regulated by c-jun NH2-terminal kinase. *Journals of the American Society of Nephrology*, 16(6), 1603–1611.
- Goda S, Kato Y, Domae E, Hayashi H, Tani-Ishii N, Iida J, et al. (2015). Effects of JNK1/2 on the inflammation cytokine TNF-alpha-enhanced production of MMP-3 in human dental pulp fibroblast-like cells. *International Endodontic Journal*, 48(12), 1122–1128. [PubMed: 25393585]
- Gronthos S, Mankani M, Brahimi J, Robey PG, & Shi S (2000). Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America*, 97(25), 13625–13630. [PubMed: 11087820]
- Hall BE, Zhang L, Sun ZJ, Utreras E, Prochazkova M, Cho A, et al. (2016). Conditional TNF-alpha overexpression in the tooth and alveolar bone results in painful pulpitis and osteitis. *Journal of Dental Research*, 95(2), 188–195. [PubMed: 26503912]
- Heinrich PC, Castell JV, & Andus T (1990). Interleukin-6 and the acute phase response. *The Biochemical Journal*, 265(3), 621–636. [PubMed: 1689567]
- Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, et al. (2002). A central role for JNK in obesity and insulin resistance. *Nature*, 420(6913), 333–336. [PubMed: 12447443]
- Hu Y, Lou J, Mao YY, Lai TW, Liu LY, Zhu C, et al. (2016). Activation of MTOR in pulmonary epithelium promotes LPS-induced acute lung injury. *Autophagy*, 12(12), 2286–2299. [PubMed: 27658023]
- Kobayashi Y (2008). The role of chemokines in neutrophil biology. *Frontiers in Bioscience*, 13(1), 2400–2407. [PubMed: 17981721]
- Kokkas AB, Goulas A, Varsamidis K, Mirtsou V, & Tziafas D (2007). Irreversible but not reversible pulpitis is associated with up-regulation of tumour necrosis factor alpha gene expression in human pulp. *International Endodontic Journal*, 40(3), 198–203. [PubMed: 17305696]
- Lamkanfi M, & Dixit VM (2014). Mechanisms and functions of inflammasomes. *Cell*, 157(5), 1013–1022. [PubMed: 24855941]
- Lee SI, Min KS, Bae WJ, Lee YM, Lee SY, Lee ES, et al. (2011). Role of SIRT1 in heat stress- and lipopolysaccharide-induced immune and defense gene expression in human dental pulp cells. *Journal of Endodontics*, 37(11), 1525–1530. [PubMed: 22000456]

- Lee YH, Lee HY, Kim TG, Lee NH, Yu MK, & Yi HK (2015). PPARgamma maintains homeostasis through autophagy regulation in dental pulp. *Journal of Dental Research*, 94(5), 729–737. [PubMed: 25744070]
- Matsuzawa-Ishimoto Y, Hwang S, & Cadwell K (2018). Autophagy and inflammation. *Annual Review of Immunology*, 36(1), 73–101.
- Mente J, Geletneky B, Ohle M, Koch MJ, Friedrich Ding PG, Wolff D, et al. (2010). Mineral trioxide aggregate or calcium hydroxide direct pulp capping: An analysis of the clinical treatment outcome. *Journal of Endodontics*, 36(5), 806–813. [PubMed: 20416424]
- Morselli E, Marino G, Bennetzen MV, Eisenberg T, Megalou E, Schroeder S, et al. (2011). Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome. *The Journal of Cell Biology*, 192(4), 615–629. [PubMed: 21339330]
- Nguyen C, Savouret JF, Widerak M, Corvol MT, & Rannou F (2017). Resveratrol, potential therapeutic interest in joint disorders: A critical narrative review. *Nutrients*, 9(1) pii: E45. [PubMed: 28067817]
- Nunes S, Danesi F, Del Rio D, & Silva P (2017). Resveratrol and inflammatory bowel disease: The evidence so far. *Nutrition Research Reviews*, 31(1), 85–97. [PubMed: 29191255]
- Park D, Jeong H, Lee MN, Koh A, Kwon O, Yang YR, et al. (2016). Resveratrol induces autophagy by directly inhibiting mTOR through ATP competition. *Scientific Reports*, 6, 21772. [PubMed: 26902888]
- Paula-Silva FW, Ghosh A, Silva LA, & Kapila YL (2009). TNF-alpha promotes an odontoblastic phenotype in dental pulp cells. *Journal of Dental Research*, 88(4), 339–344. [PubMed: 19407154]
- Roebuck KA (1999). Regulation of interleukin-8 gene expression. *Journal of Interferon & Cytokine Research*, 19(5), 429–438.
- Rubinsztein DC, Marino G, & Kroemer G (2011). Autophagy and aging. *Cell*, 146(5), 682–695. [PubMed: 21884931]
- Smith AJ, Smith JG, Shelton RM, & Cooper PR (2012). Harnessing the natural regenerative potential of the dental pulp. *Dental Clinics of North America*, 56(3), 589–601. [PubMed: 22835540]
- Takanche JS, Kim JS, Kim JE, Han SH, & Yi HK (2018). Schisandrin C enhances odontoblastic differentiation through autophagy and mitochondrial biogenesis in human dental pulp cells. *Archives of Oral Biology*, 88(4), 60–66. [PubMed: 29407753]
- Tang HW, Liao HM, Peng WH, Lin HR, Chen CH, & Chen GC (2013). Atg9 interacts with dTRAF2/ TRAF6 to regulate oxidative stress-induced JNK activation and autophagy induction. *Developmental Cell*, 27(5), 489–503. [PubMed: 24268699]
- Timmers S, Hesselink MK, & Schrauwen P (2013). Therapeutic potential of resveratrol in obesity and type 2 diabetes: New avenues for health benefits? *Annals of the New York Academy of Sciences*, 1290(1), 83–89. [PubMed: 23855469]
- Toubal A, Treuter E, Clement K, & Venticlef N (2013). Genomic and epigenomic regulation of adipose tissue inflammation in obesity. *Trends in Endocrinology and Metabolism*, 24(12), 625–634. [PubMed: 24169451]
- Vanden Berghe W, De Bosscher K, Boone E, Plaisance S, & Haegeman G (1999). The nuclear factor-kappaB engages CBP/p300 and histone acetyltransferase activity for transcriptional activation of the interleukin-6 gene promoter. *The Journal of Biological Chemistry*, 274(45), 32091–32098. [PubMed: 10542243]
- Wang FM, Galson DL, Roodman GD, & Ouyang H (2011). Resveratrol triggers the pro-apoptotic endoplasmic reticulum stress response and represses pro-survival XBP1 signaling in human multiple myeloma cells. *Experimental Hematology*, 39(10), 999–1006. [PubMed: 21723843]
- Woo JH, Lim JH, Kim YH, Suh SI, Min DS, Chang JS, et al. (2004). Resveratrol inhibits phorbol myristate acetate-induced matrix metalloproteinase-9 expression by inhibiting JNK and PKC delta signal transduction. *Oncogene*, 23(10), 1845–1853. [PubMed: 14661062]
- Yuan H, & Marmorstein R (2013). Biochemistry. Red wine, toast of the town (again). *Science*, 339(6124), 1156–1157. [PubMed: 23471392]

Fig. 1.

Resveratrol (RSV) inhibited TNF α -induced JNK activation but not NF- κ B activation in DPSCs. A: DPSCs were pretreated with DMSO only (0 μ M RSV), 50 μ M RSV, 100 μ M RSV for 1 h followed by TNF α treatment at 2.5 ng/mL for 0, 5 min, 30 min, and 60 min. Whole-cell protein lysates were harvested at each indicated time points and analyzed by Western blotting for observing the activation of NF- κ B pathway (I κ B α , phosphorylated and total p65) and MAPK pathway (phosphorylated and total p38, phosphorylated and total JNK). *, non-specific band. B: DPSCs were pretreated with DMSO only (0 μ M RSV) or 100 μ M RSV for 1 h followed by TNF α treatment at 2.5 ng/mL for 60 min. Cells were processed for immunofluorescent staining of p65 (green) and counterstaining of F-actin (Phalloidin, red) and nucleus (DAPI, blue). Representative images from confocal microscopy were shown. Scale bar, 25 μ m (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 2.

Both resveratrol and JNK inhibitor SP600125 inhibited TNF α -induced *IL6* and *IL8* mRNA expression in DPSCs. A and B: DPSCs were pretreated with DMSO (0 μ M RSV), 50 μ M RSV, 100 μ M RSV for 1 h followed by TNF α treatment at 2.5 ng/mL for 6 h. RNA was extracted from each sample and real-time reverse transcription PCR was performed to analyze the levels of *IL6* mRNA (A) and *IL8* mRNA (B). C and D: DPSCs were pretreated with DMSO (0 μ M SP600125), 50 μ M SP600125 for 1 h followed by TNF α treatment at 2.5 ng/mL for 6 h. RNA was extracted from each sample and real-time reverse transcription PCR was performed to analyze the levels of *IL6* mRNA (C) and *IL8* mRNA (D). All data are presented as mean $\pm$ SEM. Relative mRNA levels were normalized to the DMSO group without TNF α treatment. * p < 0.05 and ** p < 0.01 compared to the DMSO group as determined by Student's t -test; n.s., not significant.

Fig. 3.

Resveratrol induced autophagy which regulated TNF α -induced *IL6* and *IL8* mRNA expression in DPSCs. A: The same protein samples as analyzed in Fig. 1 were analyzed by Western blotting for observing autophagic membrane-bound form LC3B (LC3B-II) with β -actin as a loading control. B: DPSCs were treated with or without 50 μ M RSV for 2 h and processed for immunofluorescent staining of autophagic puncta (LC3B, green) and counterstaining of F-actin (Phalloidin, red) and nucleus (DAPI, blue). Representative images from confocal microscopy were shown. Scale bar, 10 μ m. C: Gene silencing of *Atg5* was performed by transfecting control siRNA (Con siRNA) or *Atg5* siRNA into DPSCs. Whole-cell protein lysates were harvested 24 h after transfection and analyzed by Western blotting for ATG5, LC3B, phosphorylated and total JNK, and β -actin (loading control). *, non-specific band. D and E: After gene silencing of *Atg5*, DPSCs were exposed to TNF α at 2.5 ng/mL for 6 h. RNA was extracted from each sample and real-time reverse transcription PCR was performed to analyze the levels of *IL6* and *IL8* mRNA. All data are presented as mean $\pm$ SEM. Relative mRNA levels were normalized to the group without TNF α treatment for each set of siRNA transfected cells. * $p < 0.05$ and ** $p < 0.01$ compared to the control siRNA group as determined by Student's *t*-test (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Dental pulp stem cells

Fig. 4.

Schematic representation of TNF α signaling regulation by resveratrol in DPSCs.

Resveratrol suppresses the TNF α -JNK pathway, perhaps by activating autophagy. Decreased JNK activation leads to down-regulation of the TNF α -induced expression of inflammatory response genes, such as *IL6* and *IL8*.