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Autophagy Modulates the Expression of Interferon Regulatory Factor 8 in Apical Periodontitis

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ABSTRACT

Objectives: *This study aims to investigate how autophagy regulates interferon regulatory factor 8 (IRF8) and other inflammation-related genes in apical periodontitis.*

Materials and Methods: *Autophagy was induced and inhibited in human leukemia monocytic cell line THP-1 and macrophages via the treatment of various concentrations of rapamycin and 3-Methyladenine (3-MA), respectively. The cytotoxicity and proliferation of the cells under these treatments were assessed using the cell counting kit 8 (CCK8) assay, and optimum concentrations of rapamycin and 3-MA were determined. Changes in the expression of IRF8 and other key markers of autophagy were measured at the transcription and protein levels via quantitative real-time polymerase chain reaction and Western Blot. Total RNAs from those cells were also subject to RNA sequencing, bioinformatics, and statistical analyses. The differentially expressed genes, especially in IRF8 and inflammation-related pathways, were analyzed.*

Results: *IRF8 was significantly downregulated at both transcription and protein levels upon inhibition of autophagy (P< 0.05, one-way ANOVA). In addition, pro-inflammatory factors involved in apical periodontitis, such as TNF-α and IL1-β, were significantly upregulated upon inhibition of autophagy. Moreover, RNA sequencing revealed many differentially expressed genes, including IRF8 and its related inflammatory pathways.*

Conclusions: *Autophagy regulates the expressions of IRF8 and related genes, which may play a pivotal role in modulating apical periodontitis.*

Clinical Relevance: Dissecting the interrelationship between autophagy and IRF8 is essential to understanding the disease pathogenesis in apical periodontitis and may lead to the development of adjunct therapies in endodontics.

Keywords

Autophagy, Apical periodontitis, Inflammation, IRF8, Macrophages.

Introduction

Bacterial infection of the dental pulp can propagate through the root canal system, resulting in the development of periapical lesions characterized by the destruction of periradicular bone and surrounding tissues [1-3]. The inflammatory process, known as apical periodontitis, is characterized by a dynamic interaction between root canal microbes and host defense mechanisms [1-3]. As microbes invade deeper into the root canal system, the host initiates a defense response involving various types of cells, such as polymorphonuclear leukocytes and macrophages [4-6]. Inflammatory mediators and cytokines have also been shown to play a significant role in the pathogenesis and progression of apical periodontitis [6]. The interaction between these factors synergistically contributes to the destruction of periapical tissues, leading to the development of apical periodontitis [6]. Unfortunately,

the body typically cannot eliminate microbes once established in the necrotic root canal system. As a result, apical periodontitis does not resolve spontaneously, necessitating endodontic intervention to reduce and eradicate microbial infection in order to promote the healing of the apical bone. Therefore, it is crucial to understand the complexity of individual host immune responses, as they can influence the pathogenicity of apical periodontitis and potentially contribute to failures in endodontic treatment and the formation of periapical lesions [7].

Autophagy has been documented in human periapical lesions [8]. Autophagy is an evolutionarily conserved process that breaks down damaged proteins and organelles in the cytoplasm and recycles them for essential cellular functions [9-12]. In non-stressed cells, autophagy functions to maintain cellular homeostasis. In contrast, in cells experiencing stress, such as starvation, autophagy is critical in providing energy through the orderly degradation of cellular components [13-15]. Studies have shown autophagy can serve dual roles: it can provide nutrients and energy to support cell survival, while conversely, it can also promote autophagic cell death. The specific role that autophagy assumes, whether protectively or destructively, depends on various factors, including the cellular context within the microenvironment and the severity of the stress encountered [11,12]. Additionally, autophagy has been shown to influence innate and adaptive immunity, inflammation, and apoptosis [11,12,14,15]. Therefore, dysregulation of autophagy has been associated with the pathogenesis of numerous diseases. Interestingly, through immunofluorescence and transmission electron microscopy techniques, autophagy has been identified in human periapical lesions diagnosed as radicular cysts or periapical granulomas [8]. However, the exact mechanisms by which autophagy functions within these lesions remain unclear.

Recently, a vital interferon regulatory factor (IRF), IRF8, has been identified in human periapical lesions [16]. Initially recognized for its critical role in differentiating myeloid cell lineages [17,18], IRF8 is induced by interferons during innate immune responses against pathogens and functions as a transcription factor that can activate or suppress genes involved in host defense mechanisms [19-24]. Notably, many studies have demonstrated that IRF8 plays an essential role in differentiating and activating macrophages during immune response [25-27]. Using immunohistochemistry, Yu et al. detected IRF8 in human periapical tissues diagnosed with radicular cysts and periapical granulomas, revealing significantly higher levels than in healthy human periapical tissues [16].

Based on the coexistence of autophagy and IRF8-related cellular processes in human periapical lesions, it is plausible to speculate about their interrelationship. Immunohistochemistry studies have confirmed the colocalization of IRF8 and LC3B, a biomarker for autophagy [16]. Moreover, IRF8 has been shown to activate multiple autophagy-related genes in macrophages directly, and studies using *Irf8-/-* macrophages have demonstrated their impaired autophagic capabilities [28]. However, the regulatory relationship between autophagy and IRF8, mainly whether a feedback mechanism exists from autophagy to IRF8, remains unclear, especially in the context of apical periodontitis. Therefore, we hypothesized that IRF8 expression is regulated by autophagy in periapical tissues. Given that macrophages are prominent in both autophagy and periapical lesions [4,29], we utilized the leukemia monocytic cell line THP-1 and macrophage cell lines as an *in vitro* platform to study how autophagy influences IRF8 in apical periodontitis [30]. Here, we analyzed the effects of autophagy stimulation and inhibition on the IRF8 gene and protein expression levels. Additionally, we identified differentially expressed genes associated with apical periodontitis. Our results facilitated our understanding of disease pathogenesis and could potentially contribute to developing new therapeutic approaches in endodontics to promote the healing of apical lesions.

Materials and Methods

Cell culture

Human THP-1 and macrophage cell lines (ATCC, Manassas, VA) were cultured in RPMI-1640 Medium (Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum (FBS; Gibco, Life Technologies, Carlsbad, CA) and 1% L-glutamine/penicillin/streptomycin solution (Gemini Bio-Products) at 37°C in 5% CO_2.

CCK-8 assays

In order to determine the optimal concentration for use in subsequent experiments, 3-Methyladenine (3-MA) was initially diluted to 1mM, 2.5mM, and 5mM, and rapamycin was initially diluted to 0.2μM, 1μM, and 5μM, respectively. Cell proliferation and cytotoxicity were determined using the cell counting kit-8 (CCK-8) assays (Gibco, Life Technologies Carlsbad, CA). THP-1 cells were pre-incubated in a 96-well plate (5,000 cells/well) for 24 hours. 3-MA and rapamycin were then added into the culturing medium and incubated for 24, 48, 72, and 96 hours, respectively. At the end of each incubation period, 10μl of CCK-8 solution was added to each well and further incubated for 4 hours in the incubator. The absorbance at 450 nm was measured using a microplate reader.

mRNA expression assays

THP-1 cells (0.5×10^5 cells/ml) were incubated in a 12-well plate (1 ml/well) for 24 hours and then treated with the culture medium with or without 2.5mM 3-MA or 5μM rapamycin respectively for another 24 hours. Total RNA was extracted using an RNAeasy kit (Qiagen, Germantown, MD). RNA quality was analyzed using 2100 Bioanalyzer (Agilent Technologies), and complementary DNA was synthesized using 3μg RNA/sample as a template in a reverse-transcription reaction (QuantiTectRT Kit, Qiagen, Germantown, MD). Quantitative real-time polymerase chain reaction (RT-PCR) was used for mRNA expression analysis of *GAPDH*, *IRF8*, *TNFA*, *IL1B*, and *TGFB1*, which was performed in an ABI Viia7 instrument using SYBR Green chemistry (Applied Biosystems Carlsbad, CA), 100nM of specific primers, and 2.5ng cDNA in each reaction. Primer sequences were designed using Primer Express software (Applied Biosystems, Carlsbad, CA). The standard PCR conditions were 95°C (10 minutes), followed

by 40 cycles of 94°C (1 minute), 56°C (1 minute), and 72°C (2 minutes). Expression levels of each target gene were normalized to the expression of *GAPDH* in each sample and analyzed using the $2^{-\Delta\Delta Ct}$ method. Statistical differences between groups were assessed using GraphPad PRISM software version 8.0 (GraphPad, San Diego, CA). Statistical analyses were performed using two-way ANOVA and Tukey's multiple comparisons tests. $P<0.05$ was considered statistically significant. Experiments were run in triplicates. Results are presented as fold-change in comparison with controls (untreated cells).

RNA Sequencing

THP-1 cells (0.5 x 10^5 cells/ml) were incubated in a 12-well plate (1 ml/well) for 24 hours and then treated with the culture medium with or without 2.5mM 3-MA or 5µM rapamycin respectively for another 24 hours. Total RNA was isolated using the PureLink RNA Mini Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA). Triplicates of 1µg of total RNA of each group were submitted for Poly(A) RNA sequencing services (RNA-Seq, LC Sciences LLC, Houston, TX). StringTie was used to perform expression level analysis for mRNAs by calculating fragments per kilobase of transcript per million mapped reads (FPKM) [31]. The differentially expressed mRNAs were selected with log2(fold change) > 1 or log2(fold change) < -1 and with statistical significance ($P<0.05$) by R package edgeR [32]. GO [33] and KEGG [34] were also performed accordingly.

Western Blot

THP-1 cells (0.5 x 10^5 cells/ml) were incubated in a 12-well plate (1 ml/well) for 24 hours and then treated with the culture medium with or without 2.5mM 3-MA or 5µM rapamycin respectively for another 24 hours. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (NaCl 150 mM, Tris pH-7.4 50 mM, Nonidet P-40 1%, and SDS 0.1%) and submitted for western blot analysis using IRF8 antibodies. β-actin was used as the control.

Results

Optimum concentrations of 3-MA and rapamycin were determined for THP-1 cells

3-MA [35] and rapamycin [36] were used to inhibit and induce autophagy in THP-1 cells, respectively. In order to determine the optimum concentrations without significantly affecting cell viability and proliferation, CCK-8 assays were performed with different concentrations of 3-MA (Figure 1A, 1mM, 2.5mM, and 5mM) and rapamycin (Figure 1B, 0.2µM, 1µM, and 5µM), respectively. DMSO was used as the control. THP-1 cells did not proliferate with the treatment of 5mM 3-MA but proliferated at various rates under lower concentrations of 3-MA (Figure 1A). In contrast, all rapamycin groups displayed similar proliferation rates (Figure 1B). Therefore, 2.5mM 3-MA and 5µM rapamycin were used for the following assays.

Inhibition of autophagy downregulates the expression of IRF8 mRNA and proteins

THP-1 cells treated with 2.5mM 3-MA showed significantly decreased levels of the *IRF8* mRNA (0.22-fold-change, $P<0.01$, Figure 2A). Similar results were observed in macrophage cells (0.09-fold-change, $P<0.01$, Figure 2B). THP-1 cells and macrophages treated with 5µM rapamycin had increased levels of *IRF8* mRNA; however, this was not statistically significant (Figure 2A and 2B). Next, to investigate if the IRF8 protein levels were also changed, western blots were performed on THP-1 cells treated with 2.5mM 3-MA and 5µM rapamycin, respectively (Figure 2C), and the results were further quantified and shown in the bar graph (Figure 2D). Similar to the mRNA changes, 3-MA treatment led to significantly decreased IRF8 protein levels (0.2-fold-change, $P<0.01$, Figure 2C and 2D). THP-1 cells treated with 5µM rapamycin had increased levels of IRF8 protein levels; however, this was not statistically significant (Figure 2C and 2D).

Inhibition of Autophagy Upregulates the Expression of *TNFA* and *IL1B* mRNAs

To further assess whether induction and/or inhibition of autophagy is involved in apical periodontitis, the mRNA levels of key cytokines and growth factors were also examined upon treatment of 2.5mM 3-MA and 5µM rapamycin, respectively. Interestingly, *TNFA* and *IL1B* mRNA levels were significantly upregulated upon the inhibition of autophagy by 3-MA ($P<0.01$, Figure 3A and 3B). *TGFB1* mRNA levels were upregulated upon induction of autophagy by 5µM rapamycin and downregulated upon inhibition of autophagy by 3-MA. However, the changes in *TGFB1* mRNA levels were not statistically significant (Figure 3C).

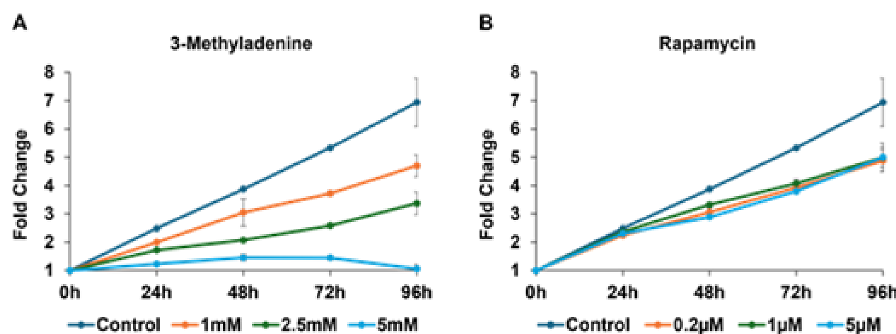

Figure 1: Cytotoxicity of various concentrations of 3-MA and rapamycin. Cell proliferation was determined using the CCK-8 assay. THP-1 cells were pre-incubated in a 96-well plate for 24 hours. Different concentrations of (**A**) 3-MA (1mM, 2.5mM, and 5mM) and (**B**) rapamycin (0.2µM, 1µM, and 5µM) were then added into the culturing medium and incubated for 24, 48, 72, and 96 hours, respectively. At the end of each incubation period, 10µl of CCK-8 solution was added to each well and further incubated for 4 hours in the incubator. The absorbance at 450 nm was measured using a microplate reader.

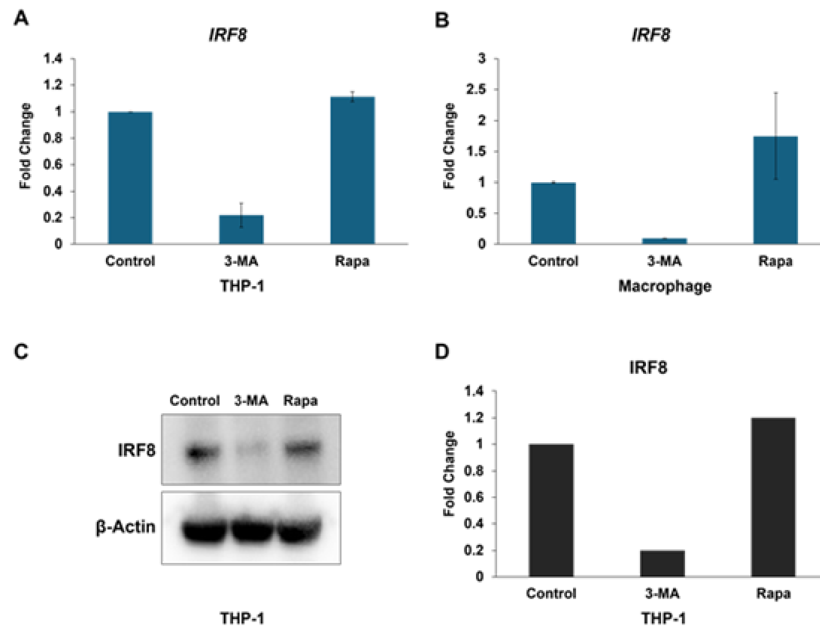

Figure 2: Fold changes of mRNA expression and protein levels of IRF8 in response to 3-MA and rapamycin. THP-1 cells (**A**) and macrophages (**B**) were treated with 2.5mM 3-MA or 5µM rapamycin, respectively. Total RNAs were extracted, and qRT-PCR showed *IRF8* genes were significantly reduced with the treatment of 2.5mM 3-MA ($P<0.05$). Cell lysates from THP-1 cells treated with 2.5mM 3-MA or 5µM rapamycin were subjected to western blot (**C**) and quantification analysis (**D**). 3-MA treatment led to significantly decreased IRF8 protein levels (0.2-fold-change, $P<0.01$). THP-1 cells treated with 5µM rapamycin had increased levels of IRF8 protein levels; however, this was not statistically significant.

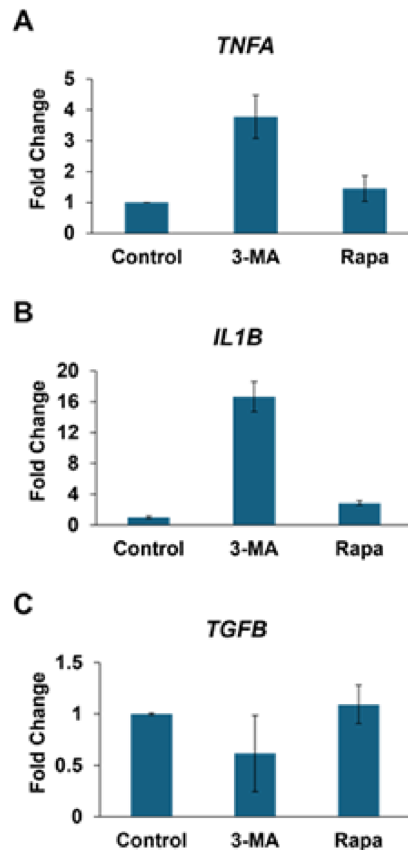

Figure 3: Fold changes of mRNA expression levels of *TNFA*, *IL1B*, and *TGFB1* in response to 3-MA and rapamycin. THP-1 cells were treated with 2.5mM 3-MA and 5µM rapamycin, respectively. Total RNAs were extracted, and qRT-PCR showed differentially expressed mRNA expression levels of *TNFA* (**A**), *IL1B* (**B**), and *TGFB*(**C**).

Autophagy affects many genes associated with IRF8 and apical periodontitis

In order to unveil all the genes that are differentially expressed upon induction and inhibition of autophagy, RNAs were purified from THP-1 cells that had been treated with either 2.5mM 3-MA or 5μM rapamycin and submitted for RNA sequencing. RNAs from DMSO-treated cells were used as the control. Differentially expressed genes were analyzed via comparisons among 3-MA, rapamycin, and control and were plotted in the heat maps (Figure 4). Notably, IRF8 was significantly downregulated when comparing the 3-MA group to the rapamycin group (Figure 4C), corroborating with the RT-PCR results (Figure 2). Moreover, many genes that are associated with inflammation and apical periodontitis were also revealed in the heat map as differentially expressed genes.

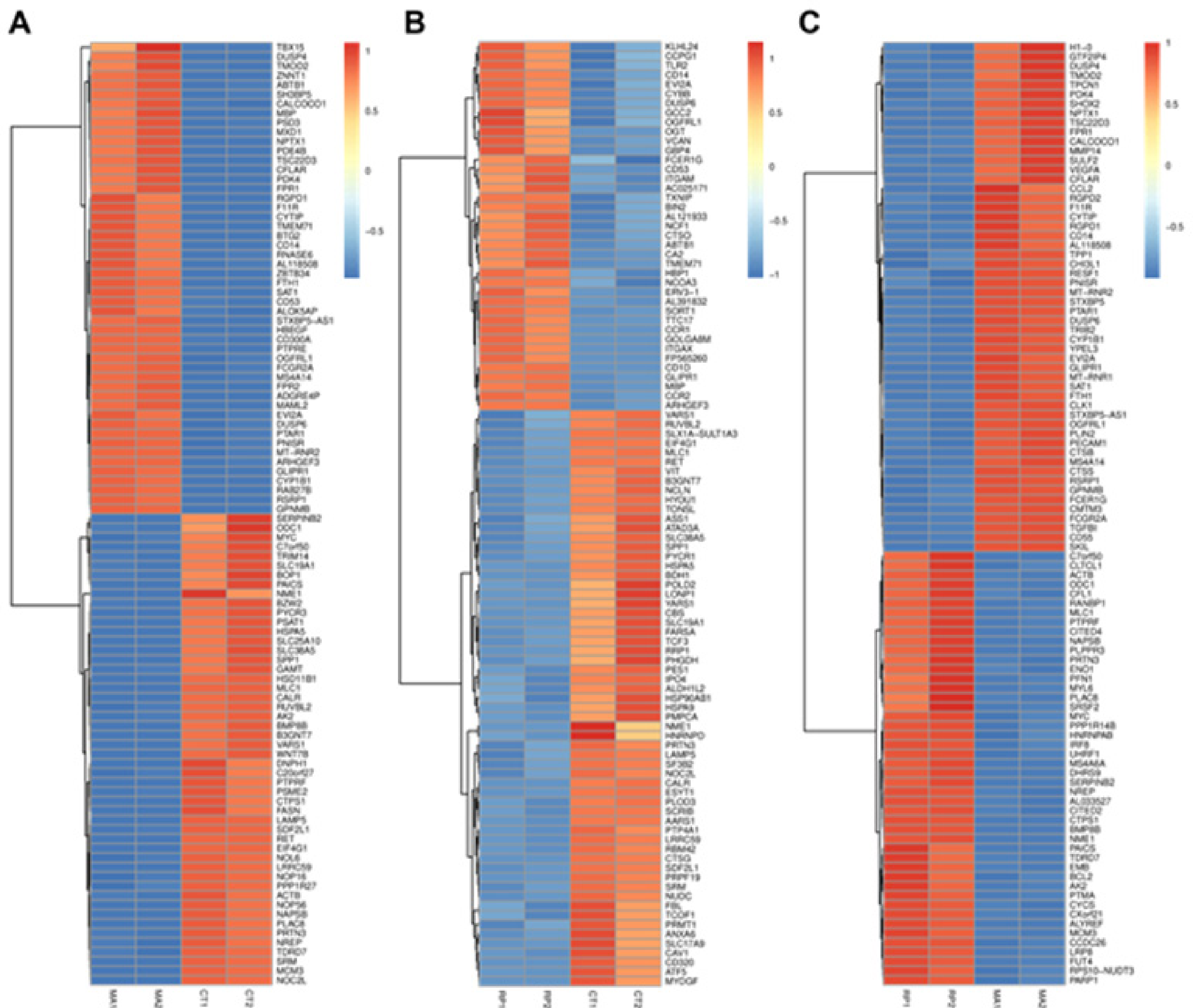

Figure 4: RNA sequencing and analysis of differentially expressed genes in response to 3-MA and rapamycin. RNAs were purified from THP-1 cells that had been treated with either 2.5mM 3-MA or 5μM rapamycin and submitted for RNA sequencing. RNAs from DMSO-treated cells were used as the control. Differentially expressed genes were analyzed via comparisons among 3-MA, rapamycin, and control and were plotted in the heat maps. (**A**) 3-MA vs. Control; (**B**) Rapamycin vs. Control; and (**C**) 3-MA vs. Rapamycin.

Discussion

Autophagy has been linked to human apical diseases from multiple reports [8,37,38]. In one study, the autophagic marker LC3 was

immunolabeled in clinical samples of radicular cysts and periapical granulomas, and immunohistochemical analysis showed that LC3 was extensively expressed in both inflamed tissue samples compared to healthy tissues [8]. Furthermore, several groups proposed mechanisms for how autophagy regulates apical periodontitis. For example, Lai et al. reported that autophagy may have a positive effect against apical periodontitis by inhibiting the apoptosis of osteoblasts [37]. Wu et al. discovered that autophagy may also play a positive role by regulating the RANKL/RANK/OPG/osteoclasts pathways to inhibit excessive bone loss in apical periodontitis [38]. Our study demonstrated that inducing and inhibiting autophagy can upregulate and downregulate the expression of IRF8 mRNA and proteins, respectively. This suggests that in addition to modulating osteoblast and osteoclast activities, autophagy may regulate the inflammatory process in apical periodontitis via IRF8-mediated molecular pathways.

On the other hand, the significant role that IRF8 plays in regulating autophagy was also revealed. Yu et al. found that IRF8 was widely expressed in radicular cysts and periapical granulomas [16]. In this study, IRF8 was localized in CD68+ macrophages, indicating its potential regulatory nature in periapical diseases [16]. In addition, Gupta et al. demonstrated that *Irf8-/-* macrophages were deficient in autophagic activity, and IRF8 played a direct role in aiding the formation of autophagosome and lysosomal function by activating autophagy-related genes [28]. These data suggest that autophagy and IRF8 may have a synergistic effect during immune response, either through induction by inflammatory mediators and cytokines or from a positive feedback mechanism between each other.

Our RNA sequencing results showed that the IRF8 gene expression was significantly downregulated when comparing the 3-MA group to the rapamycin group (Figure 4C), corroborating with the RT-PCR results (Figure 2). In addition, a list of other genes that were differentially expressed upon induction/inhibition of autophagy was also discovered, which may further provide mechanistic insights into how autophagy functions in apical periodontitis. For instance, BCL2 was significantly downregulated when autophagy was inhibited, indicating that the apoptosis pathways may be involved, similar to the findings reported by Lai et al. [37]. Another example comes from the altered MYC mRNA levels as shown in Figure 4C. As transcription factors, MYC proteins regulate a diverse set of cellular processes, including inflammation and autophagy [39]. Therefore, autophagy may impact the expression of the MYC family proteins that, in turn, regulate their downstream effectors. Validating the individual genes and dissecting their functions will be of great value to understanding autophagy in apical periodontitis.

Our study showed that inhibition of autophagy significantly downregulated IRF8 at both mRNA and protein levels; however, upregulation of autophagy only led to a moderate increase in IRF8 levels. One possibility is that in our current *in vitro* cell-line platform, pharmacological induction of autophagy was not robust enough without stress or immune response. Another explanation could be the broader effect of rapamycin. The interaction between rapamycin and its mammalian target of rapamycin (mTOR) complex induces many downstream signaling pathways, which further leads to a repertoire of cellular processes [40,41]. Therefore, there could be some compensatory mechanisms in our *in vitro* system. To understand the precise function of IRF8 during the cellular process of autophagy, conditional knockout of IRF8 and an *in vivo* mouse model for apical periodontitis could be utilized for future investigations [42].

Conclusion

Autophagy regulates the expression of IRF8 and other cellular pathways related to apical periodontitis. Understanding its molecular function may help develop therapeutic targets in conjunction with conventional endodontic therapies to promote the healing of apical periodontitis.

Grant

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