Journal of Immunology Research

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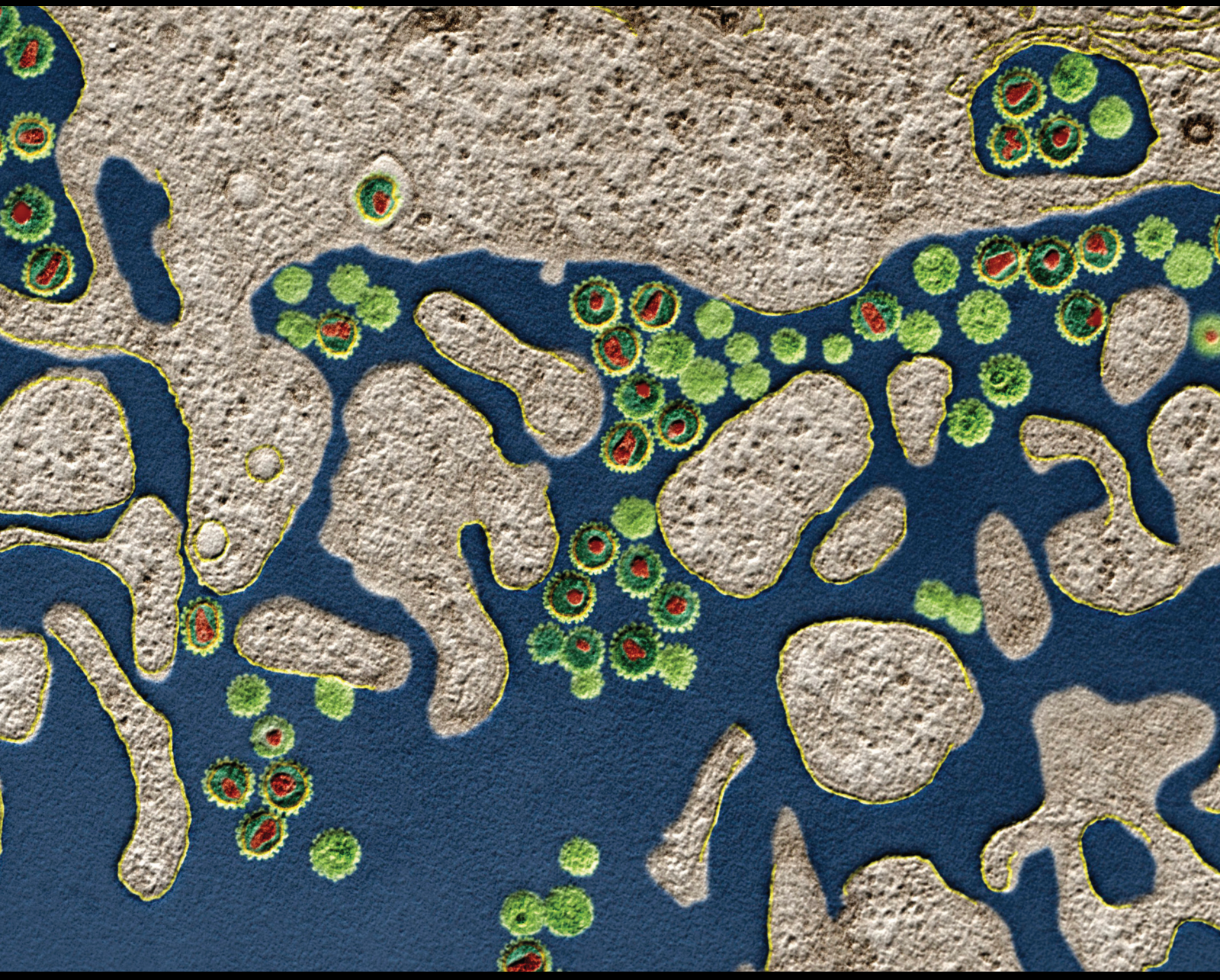

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Research Article

Changes in IL-16 Expression in the Ovary during Aging and Its Potential Consequences to Ovarian Pathology

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Aging in females is not only associated with the changes in hormonal status but is also responsible for dysregulation of immune functions in various organs including ovaries. The goal of this study was to determine whether the expression of interleukin 16 (IL-16), a proinflammatory and chemoattractant cytokine, changes during ovarian aging, to determine factors involved in such changes in IL-16 expression, and to examine if changes in IL-16 expression during aging predisposes the ovary to pathologies. Ovarian tissues from premenopausal women (30-50 years old), women at early menopause (55-59 years old), and late menopause (60-85 years old) were used. In addition, tumor tissues from patients with ovarian high-grade serous carcinoma at early stage ($n = 5$) were also used as reference tissue for comparing the expression of several selected markers in aging ovaries. The expression of IL-16, frequency of macrophages (a source of IL-16) and expression of microRNA (miR) 125a-5p (a regulator of IL-16 gene) were performed by immunohistochemistry, immunoblotting, and gene expression assays. In addition, we examined changes in nuclear expression of IL-16 expression with regards to exposure to follicle-stimulating hormone (FSH) by in vitro cell culture assays with human ovarian cancer cells. The frequencies of IL-16 expressing cells were significantly higher in ovarian stroma in women at early and late menopause as compared with premenopausal women ($P < 0.0001$). Similar patterns were also observed for macrophages. Expression of miR-125a-5p decreased significantly ($P < 0.001$) with the increase in IL-16 expression during aging. Furthermore, expression of nuclear IL-16 increased remarkably upon exposure to FSH. Consequently, ovarian aging is associated with increased expression of IL-16 including its nuclear fraction. Therefore, persistent high levels of FSH in postmenopausal women may be a factor for enhanced expression of IL-16. Effects of increased nuclear fraction of IL-16 need to be examined.

1. Introduction

Aging in females is associated with a decrease in ovarian function including folliculogenesis and gonadal steroid production [1]. As the ovary ages, depletion in follicular recruitment and growth leads to the gradual withdrawal of ovarian steroid-induced negative feedback on the pituitary resulting in sustained high levels of circulatory gonadotrophin including follicle-stimulating hormone (FSH) [2, 3]. As estrogen is known to be involved in various physiological processes, decrease in its level during aging affects the growth and maintenance of its target organs [4–6]. On the other hand, sustained high levels of circulatory FSH during aging may perturb ovarian homeostatic balance facilitating the development of abnormal condition including chronic inflammation [7] and may be risk factor associated with ovarian cancer (OVCA). Moreover, estrogen is known to be associated with the enhancement and/or maintenance of immunity [8–11]. Thus, ovarian aging not only affects reproductive functions but it may also increase the

susceptibility of ovarian tissues to chronic conditions. However, the effects of aging and its mechanism(s) in the ovary including chronic conditions are not well understood. This information is critically important for the prevention of various chronic abnormalities in the ovary specially in menopausal women.

Chronic inflammation and oxidative stress as it occurs in the ovary have been proposed as hallmarks of various pathological conditions including malignancy [12–14]. Ovarian tissues are exposed to various inflammatory factors as part of physiological processes like ovulation or infection by pathogens. Ovulation has been suggested as an inflammatory event [15]. With the release of egg during ovulation, ovarian surface epithelial cells at the site of ovulatory rupture and the fimbrial surface of the fallopian tube at the site of receiving the ovulated eggs are exposed to various toxic metabolites produced in the egg due to its metabolic processes [16]. Moreover, ovulatory injury leads to the influx of immune cells at the site of rupture in the ovarian surface and at the site of receiving of ovulated eggs in the fimbria. This results in localized inflammation and increased demand for oxygen by the accumulated immune Cell, facilitating the development of hypoxia and oxidative stress [17]. Thus, inflammation and oxidative stress are prevalent in the ovary and the fimbria of the fallopian tube. Furthermore, the oviduct is open to both external and internal environments through the vagina and fimbria, respectively, predisposing the reproductive tissues to external pathogens or internal toxic byproducts of various physiological processes. Close proximity of ovarian tissues with the fimbria increases the chance of gaining entrance of pathogens to the ovaries. In addition, food-borne pathogens from a perforated gastrointestinal tract may gain entrance to the ovary via the systemic circulation. Thus, the ovarian and fimbrial tissues are exposed to various inflammatory conditions due to frequent ovulation, external pathogens, and internal toxic byproducts as well as persistent high levels of circulatory FSH. Therefore, information on the effects of exposure to these agents will be helpful to prevent ovarian abnormalities during aging.

Ovarian tissues express many cytokines [18, 19]. Cytokines are proteins secreted by many cell types including immune cells, epithelial cells, fibroblasts, and stromal cells. Cytokines are involved in the regulation of cellular growth and differentiation, homeostasis, and immune functions in normal tissues as well as in pathological conditions including tumors [18]. In normal ovaries, interleukin- (IL-) 6 and TGF-β have been suggested to be involved in follicular development by preventing follicular atresia [20, 21]. IL-1 and TNF-α have been shown to be associated with inhibition of progesterone secretion and regression of the corpus luteum [22, 23]. Ovarian follicles have been reported to produce IL-8, while IL-11 was found in the follicular fluid [24, 25]. Unfortunately, most of the studies on cytokines on ovarian function are limited to premenopausal ovaries and studies on ovarian cytokines during aging including late menopausal stage ovaries are very scanty. Recent studies have shown increased expression of IL-16 in ovarian tumors [26, 27]. However, no information is available if persistent high levels of IL-16 expression are a risk factor to develop OVCA.

IL-16 is a proinflammatory cytokine and a chemotactic factor for other immune cells to the site of inflammation [28]. Frequent exposure of ovarian and fimbrial tissues to ovulatory insults, external and internal agents (pathogenic/metabolic), and increased levels of FSH may lead to chronic inflammation in these issues and may induce increased expression of IL-16. Chronic inflammation is a hallmark of cancer development. However, it is unknown if the expression of IL-16 increases during aging in ovaries and fimbria, and whether persistent high levels of IL-16 are associated with OVCA development. The goal of this study was to examine whether IL-16 expression increases in ovarian and fimbrial tissues during aging and whether such increase in IL-16 expression is associated with increased risk of OVCA in postmenopausal women.

2. Material and Methods

2.1. Clinical Specimens. Archived premenopausal and postmenopausal ovarian tissues from healthy/normal subjects and their blood samples were collected from the Department of Pathology Rush University Medical center, Chicago, IL. All specimens were collected under the Institutional Review Board (IRB) of the Rush University Medical Center approved protocol. These subjects underwent surgery for nonovarian cause. Ovarian tumor tissues ($n = 3$, ovarian high-grade serous carcinoma (HGSC), used as positive control) were collected from patients underwent surgery following the diagnosis of suspected ovarian mass.

Representative normal (healthy) specimens were divided into 3 groups, namely, premenopausal (30-50 years old, $n = 8$), early menopausal (55-59 years old, $n = 7$), and late menopausal (60-85 years old, $n = 9$).

2.2. Processing of Tissue Specimens. Ovarian tissues were processed for paraffin and/or frozen embedding, protein extraction, and gene expression studies. Briefly, upon receiving, tissues were washed with phosphate-buffered solution (PBS) and divided into four pieces including for paraffin and frozen sections, protein, and total RNA extraction. For paraffin embedding, tissue specimens were treated with neutral buffered formalin for 72 hours followed by washing with running water overnight, cut into blocks of desired sizes, dehydrated by treating with an ascending series of ethanol and xylene, and embedded in paraffin. The portion of fresh tissues for RNA extraction w**as** treated with RNA later (RNAlater™ Stabilization Solution, Thermofisher, Waltham, MA) and stored at -80°C for later use. For frozen sections, portions of fresh tissues were embedded in OCT compound (Miles Inc., Elkhart, IN) and snap-frozen in a mixture of methanol and solid carbon dioxide and stored at -80°C for later use. The portion of fresh tissues for protein extraction w**as** stored at -80°C for later use. Serum samples were separated from blood and stored at -80°C for further use.

2.3. Routine Stain. Sections (5 μm) were made from paraffin embedded tissue blocks and stained with hematoxylin and

eosin, examined under a light microscope by a board-certified pathologist for the presence any abnormality.

2.4. Immunohistochemical Studies

2.4.1. Specimens. Paraffin-embedded 5 μm sections were used to determine the expression of IL-16 (8 premenopausal ovaries, 7 early menopausal ovaries, and 9 late menopausal ovaries), macrophages (6 premenopausal ovaries, 6 early menopausal ovaries, and 5 late menopausal ovaries) and follicle-stimulating hormone receptor (FSHR) ($n = 5$ from each group) by immunohistochemistry.

2.4.2. Antibodies and Reagents. Immunohistochemical examinations were performed using antihuman IL-16 (Abcam, Cambridge, MA, 1 : 100 dilution), antihuman FSHR (Abcam, Cambridge, MA, 1 : 100 dilution), or antihuman macrophages primary antibodies (Thermo Fisher Scientific, Waltham, MA, 1 : 100 dilution).

2.4.3. Immunohistochemistry (IHC). IHC was carried out as reported earlier [26] using primary antibodies and other reagents as per the instructions of the manufacturers. Briefly, sections were deparaffinized with xylene and a graded series of alcohol followed by washing briefly in deionized (DI) water. Antigens on the section were unmasked by heating the sections with sodium citrate-containing antigen retrieval solution. Sections were then cooled at room temperature in phosphate-buffered solution (PBS) followed by blocking of endogenous peroxidase using 0.03% H_2O_2-containing methanol at ice-cold condition. Sections were then rinsed in PBS and treated with normal horse serum for 15 min to block endogenous nonspecific bindings using VECTASTAIN ABC Kit (Vector laboratories, INC., Burlingame, CA). Sections were then incubated overnight at 4°C with primary antibodies mentioned above. Following washing 5 min X 3 with PBS, sections were incubated at room temperature with secondary antibodies and avidin/biotinylated enzyme complex (VECTASTAIN ABC Kit (Vector laboratories, INC., Burlingame, CA)) one hour each and 5 min X 3 washing with PBS in between. After washing with PBS, immunoreactions on sections were visualized by incubating them with a chromogen DAB solution (3.3′-diaminobenzidine, DAB-Peroxidase Substrate Kit—Vector laboratories, INC., Burlingame, CA) while examining under a light microscope. Sections were then washed in running water followed by counterstaining with hematoxylin. After rinsing in water, sections were then dehydrated with a graded series of alcohol and xylene, mounted with mounting media, covered with cover slip, and oven dried. Control (negative) staining was carried out by omitting primary antibodies, and no staining was observed in these sections (Supplementary figure 1).

2.4.4. Counting of Immunopositive Cells or Intensity of Immunostaining. Frequency of immunopositive cells was counted, or intensities of immunostaining were determined using a light microscope attached to a computer-assisted digital imaging software (MicroSuite, version 5; Olympus Corporation, Tokyo, Japan). Counting was performed by two individuals blinded of the age or pathology of the subjects/patients. For IL-16 or macrophages, immunopositive cells in 3-5 areas with highest population in a section at 40X magnification were counted, and average of them was expressed as the frequency of IL-16-expressing cells or macrophages in 20,000 μm^2 area of the tissue as reported earlier [26]. Similarly, the intensities of FSHR expression were expressed in 20,000 μm^2 area as an arbitrary value as reported earlier with little modification [29].

2.4.5. Western Blotting. Proteins were extracted from tissue samples as reported earlier [26]. Briefly, tissue specimens were homogenized using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). Homogenized samples were then centrifuged, supernatants were collected, and protein concentrations from supernatants were measured using Bradford BioRad Protein Assay (Bio-Rad, Hercules, CA) method as reported earlier [26]. Protease inhibitor was added, and samples were stored at -80°C for later use. Three samples from each age group (including 30-50-, 55-59-, 60-69-, and 70-85-year old subjects) as well as OVCA patients were selected randomly for immunoblotting, and each sample was examined two times (2X) in immunoblotting. Briefly, a panel of 4 samples (tissue extracts), one from each age group, and a positive control (indicated below) was examined in each immunoblot. In gel electrophoresis, the same amount of protein (50 g) for each sample was loaded. Proteins were separated by one-dimensional electrophoresis as reported earlier [26], and separated proteins were transferred to the membrane. Immunoblotting of membranes was performed using anti-IL-16 mentioned above as primary antibodies (at 1 : 1000 dilution) and anti-rabbit HRP as secondary antibody. Immunoreactions on the membrane were visualized as chemiluminescence products (Super Dura West substrate; Pierce/Thermo Fisher, Rockford, IL). Images were captured by Quantity One software using Chemidoc XRS (Bio-Rad, Hercules, CA) system according to the manufacturer's recommendation as reported previously [26]. Images of 3 immunoblots were selected randomly for analysis. Intensity of signals of IL-16 protein expression in immunoblotting was determined from the images using the analysis® getIT! Software (Olympus Soft Imaging Solutions Corporation, Lakewood, CO). Signal intensities were quantified as arbitrary values and reported as mean + SEM in 20,000 μm^2 area. An ovarian HGSC specimen was used as positive control for IL-16 protein expression while β-actin protein was used as housekeeping protein.

2.4.6. Gene Expression Studies (Quantitative Real-Time Polymerase Chain Reaction). Expression of IL-16 gene and its regulator microRNA miR-125a-5p was examined in representative specimens (8 premenopausal ovaries, 6 early menopausal ovaries, and 4 late menopausal ovaries) by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted from all specimens using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation. RNA was then measured at an optical density (OD) of 260 nm and an OD of 260/

280 nm absorbance ratio ≥1.7 was used to evaluate the purity, as previously reported [30].

The expression of IL-16 messenger RNA (mRNA) and miR-125a-5p in normal ovaries and fimbriae was measured by quantitative real-time polymerase chain reaction (qRT-PCR). The human specific IL-16 primer (QT00075138) designed by QuantiTech and miR-125a-5p designed by Applied Biosystems (Foster City, CA) were used for qRT-PCR analyses. β-Actin was used as housekeeping gene in qRT-PCR experiments. Gene expression amplification was determined using the method of the differences (δ) in cycle threshold (ΔCt) for IL-16 mRNA and miR-125a-5p according to the manufacturer's recommendation. Subtracting ΔCt from each group from the average ΔCt determined the $\Delta\Delta$Ct. $2^{-\Delta\Delta Ct}$ was used to calculate the fold change in the differences in IL-16 mRNA and miR-125a-5p expression levels.

2.4.7. Treatment of Cells with FSH and Cell Fractionation. Human ovarian surface epithelium (OSE) cells were a kind gift from Dr. Hazel Lum, PhD, Department of Pharmacology, Rush University Medical Center. The OSE cells were grown in ovarian epithelium cell medium (OEpiCM) (ScienCell Research Laboratories, Carlsbad, CA) supplemented with 1% ovarian epithelial cell growth supplement (OEpiCGS, ScienCell Research Laboratories, Carlsbad, CA), 5% fetal bovine serum (FBS) (SAFC Biosciences Inc., Lenexa, KS), and 1% of antibiotic solution made of penicillin and streptomycin (P/S) (ScienCell Research Laboratories, Carlsbad, CA). OSE cells were then plated in 100 mm dishes with approximately 2.5×10^6 cells and were treated in triplicate with 4 uL of human recombinant FSH (Sigma-Aldrich, St. Louis, MO) mixed with 16 uL of media per well for 24 hours. After incubation, media was collected and saved. Cell fractionation was performed using the Cell Fractionation Kit (Abcam, Cambridge, UK) according to the manufacturer's recommendation. Cells were rinsed with PBS, trypsinized, and harvested (pellet). Pellet containing FSH-treated or untreated (control) were then resuspended in 1X Buffer A to 6.6×10^6 cells/mL and diluted by 1,000-fold using Buffer B. Cells were incubated at room temperature for 7 minutes with constant mixing, followed by centrifugation at 5,000 × g for 1 minute at 4°C. Pellet was removed and saved, while supernatant was then removed and centrifuged at 10,000 × g for 1 minutes at 4°C. The final supernatant contains fraction C (Cytosol). The saved pellet was then resuspended in Buffer A, diluted in Buffer C, and incubated at room temperature for 10 minutes with constant mixing. The suspension was centrifuged at 5,000 × g for 1 minute at 4°C. Pellet was removed and saved, while supernatant was then removed and centrifuged at 10,000 × g for 1 minutes at 4°C. The final supernatant contains fraction M (mitochondrial). The saved pellet was resuspended in Buffer A, containing fraction N (nuclear).

2.5. Statistical Analysis. Differences in the frequency of IL-16-expressing cells or macrophages or in the intensities of FSHR expression during aging were assessed by ANOVA and unpaired t-tests. Differences in the signal intensity of IL-16 protein expression in immunoblotting among different age groups were also determined by ANOVA and unpaired t-tests. All reported P values were a two-sided where $P < 0.05$ was considered significant. Statistical analysis was performed using the Prism GraphPad software.

3. Results

Ovarian H&E sections from premenopausal subjects showed preantral follicles were embedded in the stroma, while no follicle was observed in sections from early and late menopausal women (Figure 1). Cortical inclusion cysts (CIC) (Figure 2) and ovarian surface invaginations (INV) (Figure 3) were observed in ovarian sections from pre- and postmenopausal women. Differences in the histomorphology of these features among pre- and postmenopausal women at early and late stages were not observed (Figures 2 and 3). However, compared with premenopausal, CICs and INVs were more frequent in late menopausal ovaries (Figures 2 and 3).

3.1. Localization of IL-16 in Ovaries. Immunopositive IL-16 expressing cells were localized in the ovarian surface layer as well as in the stroma in all specimens examined including ovarian tissues from premenopausal, early, and late menopausal women (Figure 4). IL-16-expressing cells showed multiple morphologic sizes and shapes including small, medium, and large as well as rounded, elongated, and irregular (Figures 4(a)–4(c)). Epithelial cells in OSE, CIC, or INVs also showed positivity for IL-16 expression (Figures 4(d)–4(f)).

The frequency of IL-16-expressing cells in the ovarian stroma of premenopausal subjects was 4.0 ± 0.2 cells in 20,000 μm^2 area of the tissue. However, it increased significantly ($P < 0.0001$) to 5.9 ± 0.2 cells in 20,000 μm^2 area of the tissue in women at an early menopausal stage (Figure 5 (a)) and increased even further (7.0 ± 0.3 in 20,000 μm^2 area of the tissue) with aging in subjects at a late menopausal stage ($P < 0.0001$) (Figure 5(a)).

INVs and CICs are formed by OSE cells as a result of ovulatory ruptures. The frequency of IL-16-expressing cells in OSE was 3.6 ± 0.2 in 20,000 μm^2 of the tissue (Figure 5(b)). Compared with OSE, the frequency of IL-16-expressing cells was significantly higher ($P < 0.0001$) in the epithelial layer of CICs (4.85 ± 0.1 in 20,000 μm^2 of the tissue) and increased further (12.66 ± 0.2 in 20,000 μm^2 of the tissue) in the epithelial cells in INVs ($P < 0.0001$). Furthermore, significant differences in the frequency of IL-16-expressing cells in CICs among different age groups (4.7 ± 0.3, 5.0 ± 0.4, and 4.8 ± 0.1mean ± SEM in 20,000 μm^2 area of the tissue in premenopausal, early postmenopausal, and late postmenopausal groups, respectively) were not observed ($P < 0.72$) (Figure 5(c), left panel). Similarly, differences were not observed in the frequency of IL-16-expressing cells in INVs in ovaries in different age groups (12.6 ± 0.2, $12.3 + 0.5$, and 13.1 ± 0.4mean ± SEM in 20,000 μm^2 area of the tissue in premenopausal, early postmenopausal, and late postmenopausal groups, respectively) ($P < 0.36$) (Figure 5(c), right panel).

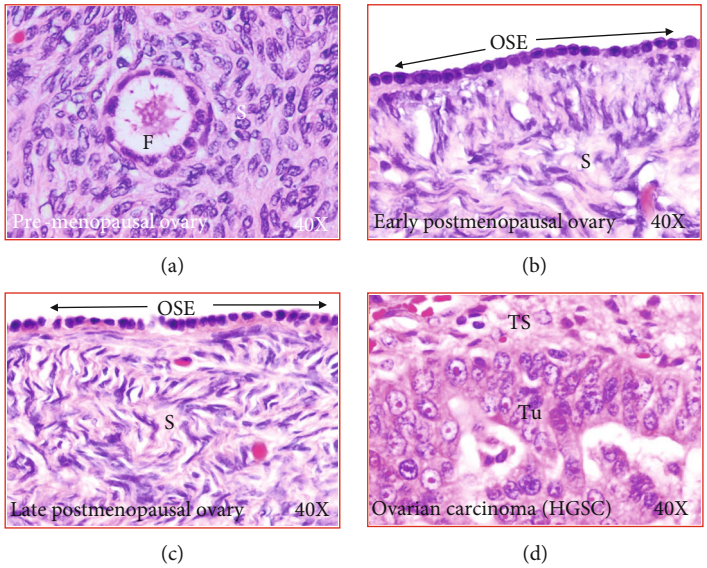

Figure 1: Microscopic presentations of healthy ovaries and ovary with cancer. (a) Section of an ovary from a premenopausal subject. An embedded follicle is seen in the ovarian stroma. (b) Section of an ovary from a healthy early postmenopausal woman showing no embedded follicle in the stroma. The ovarian surface layer is seen to be composed of rounded or flat-type of epithelial cells. (c) Section of an ovary from a healthy late postmenopausal woman showing no embedded follicle in the stroma. The ovarian surface layer is seen to be composed of rounded or flat-type of epithelial cells. (d) Section of an ovarian high-grade serous carcinoma (HGSC) at late stage. OSE: ovarian surface epithelial layer; S: stroma; TS: tumor stroma; Tu: tumor; 40×: magnification.

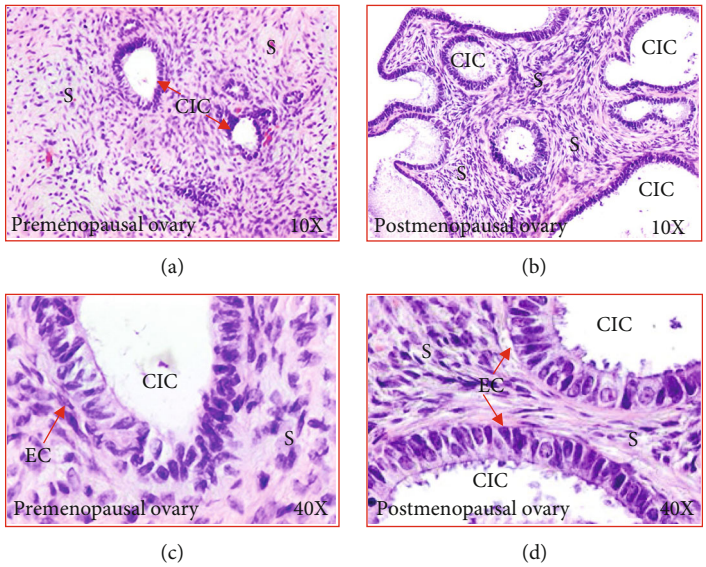

Figure 2: Cortical inclusion cysts (CIC) in healthy ovaries in pre- and postmenopausal women. (a) Section of an ovary from a premenopausal woman at low magnification (10×). Few CICs are seen in the ovarian stroma. (b) Section of an ovary from a healthy woman at late postmenopausal stage (low magnification, 10×). Compared with premenopausal woman, more CICs are seen in the ovarian stroma. (c) Section of an ovary from a healthy premenopausal woman (presented in (a)) showing a CIC in the stroma at high magnification (40×). The CICs consist of a single layer of tube-like or columnar-like epithelial cells. (d) Section of an ovary from a healthy late postmenopausal woman (presented in (b)) showing CICs in the stroma (at high magnification, 40×). As seen in the premenopausal ovary, CICs are consisted with a single layer of tube-like or columnar-like epithelial cells. EC: epithelial cells; S: stroma.

3.2. IL-16 Expression Observed by Immunoblotting. Immunoblotting studies showed a band of approximately ~60 kDa for IL-16 in all specimens examined with different intensities (Figure 6(a)). In immunoblotting, a weak or faint band for IL-16 protein was detected in specimens from premenopausal subjects (Figure 6(a)). In contrast, subjects in the early menopausal group showed strong expression for IL-16 in immunoblotting which was stronger in subjects at the late menopausal stage (Figure 6(a)). However, the intensity of IL-16 protein expression was

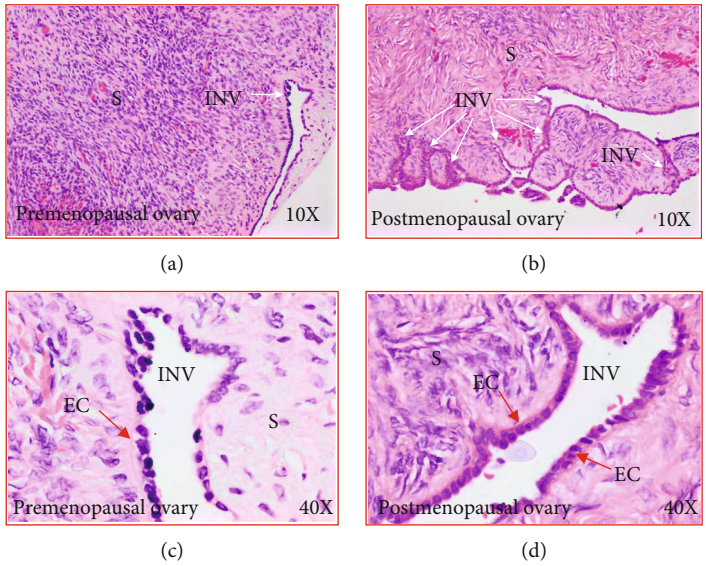

Figure 3: Stromal invaginations (INV) in healthy ovaries in pre- and postmenopausal women. (a) Section of an ovary from a premenopausal woman showing an INV in the stroma at low magnification (10×). (b) Section of an ovary from a healthy woman at late postmenopausal stage (low magnification, 10×). Compared with premenopausal woman, many INVs of different sizes and shapes are seen in the ovarian stroma. (c) Section of an ovary from a healthy premenopausal woman (presented in (a)) showing an INV in the stroma at high magnification (40×). The INV consists of a single layer of rounded or occasionally tube-like epithelial cells. (d) Section of an ovary from a healthy late postmenopausal woman (presented in (b)) showing an INV in the stroma (at high magnification, 40×). As seen in the premenopausal ovary, the INV is consisted with a single layer of rounded or occasionally tube-like epithelial cells. EC: epithelial cells; S: stroma.

weaker in subjects older than 70 years. Nuclear fraction in untreated (control) normal OSE cells showed relatively weaker expression for IL-16 (Figure 6(b)). Compared to untreated counterparts, OSE cells treated with FSH for 24 hours showed stronger expression of IL-16 (Figure 6(b)). Similar patterns of expression were also observed in ovarian HGSC cells (OVCAR3).

Compared to premenopausal ($5.9 \times 10^4 \pm 0.8 \times 10^3$ in 20,000 μm^2 area), the intensity of signal of IL-16 expression in immunoblotting was significantly higher in 58-year-old postmenopausal woman (1.13 times, $P < 0.0003$) (Figure 6(c)). Significant differences were not observed in the intensity of signals of IL-16 expression between 58-year and 68-year-old postmenopausal women ($P < 0.46$). However, compared to 68-year-old ($6.6 \times 10^4 \pm 0.9 \times 10^3$ in 20,000 μm^2 area), the intensity of signals of IL-16 expression decreased significantly in 78-year-old postmenopausal woman ($6.1 \times 10^4 \pm 0.79 \times 10^3$ in 20,000 μm^2 area) ($P < 0.01$). Nevertheless, as expected, the intensity of signals of IL-16 expression was highest in patient with ovarian HGSC (approximately 1.21 times more than 58 years old postmenopausal woman, $P < 0.03$) (Figure 6(c)). However, significant differences were not observed in the expression of β-actin among different groups ($P < 0.97$) (Supplementary figure 2).

Compared with untreated normal OSE cells ($1.14 \times 10^6 \pm 2.5 \times 10^4$ in 20,000 μm^2 area of the blot), the nuclear fraction of the OSE cells treated with FSH for 24 hours showed a significant increase in IL-16 expression ($1.28 \times 10^6 \pm 1.1 \times 10^4$ in 20,000 μm^2 area of the blot) ($P < 0.03$). Similarly, significantly higher expression of IL-16 ($1.66 \times 10^6 \pm 4.8 \times 10^3$ in 20,000 μm^2 area of the blot) ($P < 0.001$) was detected in the nuclear fraction of OVCAR3 ovarian cancer cells. However, significant differences were not observed in β-actin expression among different groups, including normal OSE cells untreated or treated with FSH for 24 hours and OVCAR3 cells ($1.67 \times 10^6 \pm 0.059 \times 10^2$ in 20,000 μm^2 area of the blot, $1.65 \times 10^6 \pm 7.78 \times 10^3$ in 20,000 μm^2 area of the blot and $1.64 \times 10^6 \pm 6.76 \times 10^3$ in 20,000 μm^2 area of the blot, respectively) (Supplementary figure 3).

3.3. Expression of IL-16 Gene and Its Regulatory MicroRNA. Expression of IL-16 gene was detected by qRT-PCR in all specimens examined (Figure 6(d)). Compared to subjects in premenopausal stage, expression of IL-16 gene increased significantly in subjects at early menopausal stage ($P < 0.001$) and even further in subjects at the late menopausal stage (Figure 6(d)).

Gene expression studies showed that increase in IL-16 gene expression during aging was inversely associated with expression of its regulatory microRNA, miR-125a-5p (Figure 6(e)). Compared with premenopausal subjects, expression of miR-125a-5p was significantly lower in women at an early menopausal stage and decreased further in subjects at the late menopausal stage (Figure 6(e)). Overall, gene expression studies supported an inverse relation between the expression of IL-16 gene and its regulator miR-125a-5p during ovarian aging.

3.4. Localization of Macrophages. Macrophages have been suggested to be a source of IL-16 production.

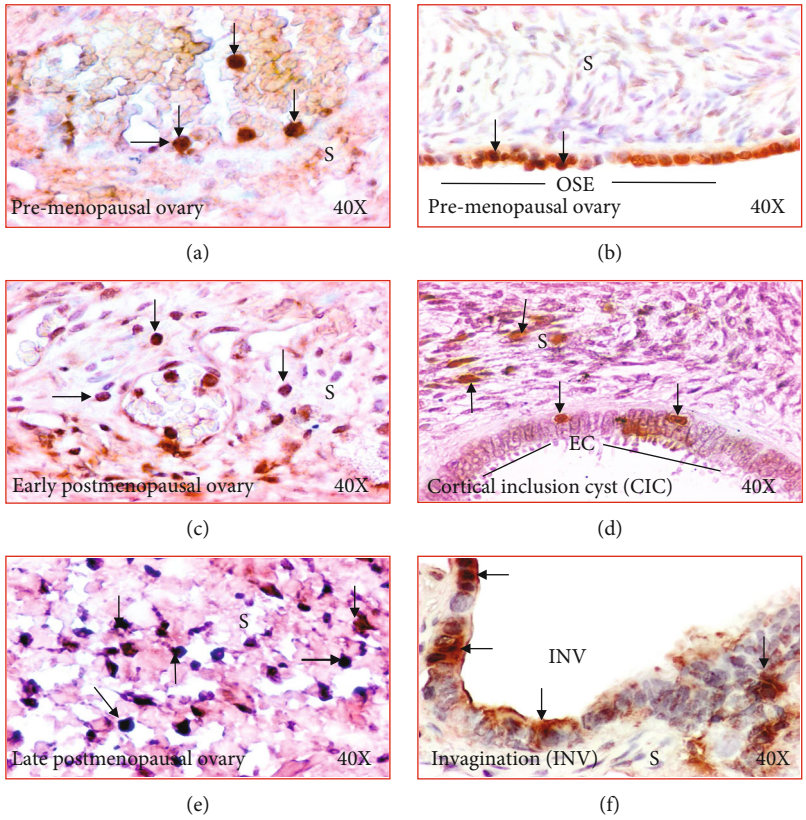

Figure 4: Changes in immunolocalization of IL-16 in human ovaries during aging. (a) Section of a premenopausal ovary showing few immunopositive IL-16-expressing cells in ovarian stroma. (b) Section of an ovary from a subject at early stage of menopause. Compared with premenopausal, more immunopositive IL-16-expressing cells are seen in the stroma. (c) Section of an ovary from a subject at late stage of postmenopause. Many immunopositive IL-16-expressing cells are localized in the stroma. (d) Section of a premenopausal ovary showing a few IL-16-expressing cells in ovarian surface epithelial (OSE) layer. (e) Section of an ovary from a subject at late menopausal stage showing IL-16 expression by the epithelial cells (EC) in a cortical inclusion cyst (CIC) in ovarian stroma. (f) Section of an ovary from a subject at late menopausal stage showing IL-16 expression by cells of stromal invagination (INV). Compared with OSE and CIC, more IL-expressing cells are seen in the epithelial cells (EC) in INV. S: stroma. Magnification = 40×.

Immunohistochemical studies detected macrophages in the stroma as well as in the vicinity of CICs and INVs, two structures formed after ovulatory rupture (Figures 7(a)–7(f)). The frequency of macrophages in the ovarian stroma in women at premenopausal stage was 4.8 ± 0.1 in 20,000 μm^2 area of the tissue (Figure 8(a)). In contrast, the frequency of macrophages was significantly higher in the ovarian stroma in women at early (5.4 ± 0.2 in 20,000 μm^2 area of the tissue) ($P < 0.05$) and late menopausal stages (6.9 ± 0.3 in 20,000 μm^2 area of the tissue) ($P < 0.0001$) (Figure 8(a)). Furthermore, compared with CICs (3.4 ± 0.2 in 20,000 μm^2 area of the tissue), the frequency of macrophages (5.8 ± 0.4 in 20,000 μm^2 area of the tissue) was significantly higher in ovarian INVs ($P < 0.0001$) (Figure 8(b)).

3.5. Expression of Follicle-Stimulating Hormone Receptors (FSHR). Menopause is one of the most remarkable physiological changes in human females and is associated with the increase in circulatory levels of FSHR. In this study, immunohistochemical studies detected FSHR expression in all ovarian specimens with different degrees of signal intensities in respective of their menopausal stage (Figure 9). In premenopausal ovaries, OSE cells showed weak to moderate intensities for FSHR expression (Figure 9(a)). In contrast, the expression of FSHR was stronger in the OSE cells in subjects at early postmenopausal stage (Figure 9(b)). Similar patterns were also seen in subjects at late menopausal stages. Furthermore, compared with CIC (Figure 9(c)), the intensity of FSHR expression was stronger in INV (Figure 9(d)).

Compared to premenopausal ($7.89 \times 10^3 \pm 1.2 \times 10^3$ in 20,000 μm^2 area of the tissue) subjects, the intensities of FSHR expression were significantly ($P < 0.04$) higher in early menopausal women ($14.25 \times 10^3 \pm 3.73 \times 10^3$ in 20,000 μm^2 area of the tissue) and increased even further in late stage ($35.07 \times 10^3 \pm 3.81 \times 10^3$ in 20,000 μm^2 area of the tissue, $P < 0.0001$) (Figure 9(e)). Furthermore, compared to CICs ($17.79 \times 10^3 \pm 3.12 \times 10^3$ in 20,000 μm^2 area of the tissue), the intensity of FSHR expression was significantly higher in INVs ($26.4 \times 10^3 \pm 1.89 \times 10^3$ in 20,000 μm^2 area of the tissue, $P < 0.0047$) (Figure 9(e)).

4. Discussion

This is the first study reporting an increase in the frequency of IL-16-expressing cells in ovaries during aging in women

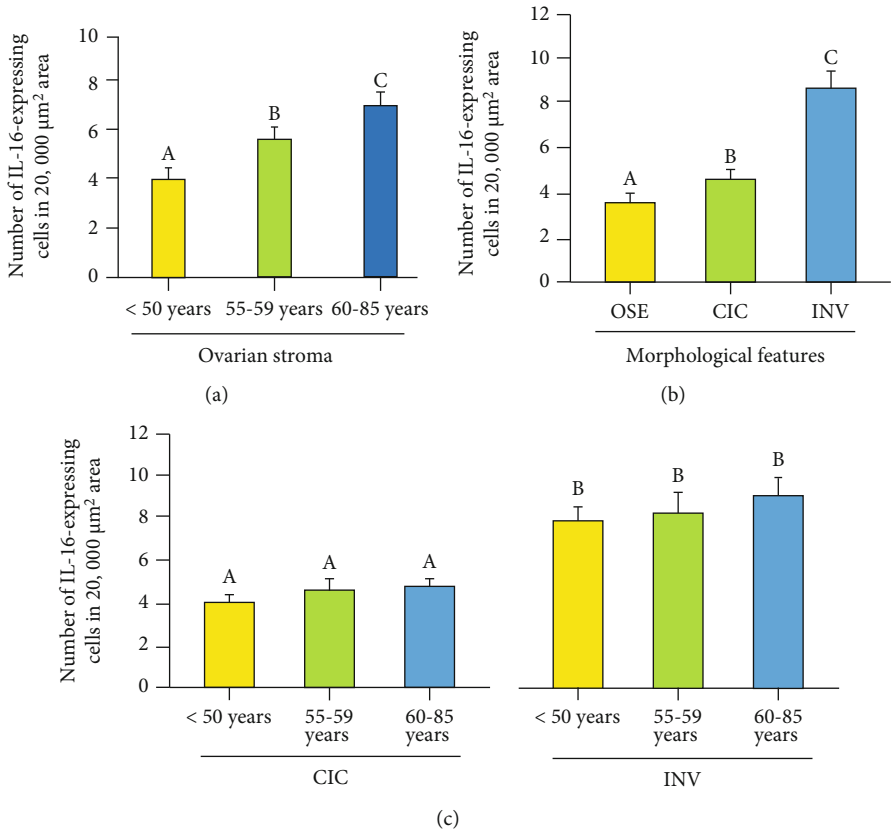

Figure 5: Changes in the frequency of IL-16-expressing cells in the ovary during aging. (a) Frequency of IL-16-expressing cells in the ovarian stroma in premenopausal and postmenopausal ovaries. Compared with premenopausal, the frequency of IL-16-expressing cells was significantly higher ($P < 0.001$) in subjects at early stage of menopause (55-59 years old) and increased further in subjects at late stage of menopause (60-85 years old). (b) Population of IL16-expressing cells in the ovarian surface epithelial (OSE) layer, cortical inclusion cysts (CICs), and stromal invaginations (INVs) in postmenopausal ovaries. Compared with OSE, the frequency of IL-16-expressing cells was significantly higher in CICs ($P < 0.0001$) and increased further in INVs ($P < 0.0001$). (c) Frequency of IL-16-expressing cells in CICs and INVs in different age groups. Significant differences were not observed in the frequency of IL-16 expressing cells in CICs among different age groups (five ovaries were randomly selected from each age group including premenopausal, early menopausal, and late menopausal stages and examined) (left panel, $P < 0.72$). Similarly, differences were not observed in the population of IL-16-expressing cells in INVs among different age groups (five ovaries were randomly selected from each age group including premenopausal, early menopausal, and late menopausal stages and examined) (right panel, $P < 0.36$). y-axis shows mean ± SEM in 20,000 mm^2 area of the tissue and bars with different letters are significantly different. Details of statistical analysis are mentioned in materials and method section of the main text.

at postmenopausal stage. This study also showed significant increase in the frequency of IL-16-expressing cells in ovarian stromal invaginations (INVs) but not in cortical inclusion cysts (CICs), a structure formed following ovulation in the ovaries. Furthermore, this study also showed that increase in IL-16 gene expression was associated with the decrease in its regulatory microRNA miR-125a-5p during aging. The increase in the frequency of IL-16-expressing cells during aging was associated with the increase in the frequency of macrophages and persistent high levels of FSH in postmenopausal women. In addition, FSH treatment of normal ovarian cells showed increased expression of nuclear IL-16. Overall, the results of this study suggest that ovarian aging is associated with prevalence of chronic stress and inflammation, and two risk factors reported to be associated with ovarian pathologies including malignancy.

This study showed that expression of IL-16 including the frequency of IL-16 expressing cells in the ovary increased significantly during aging suggesting the prevalence of chronic inflammation in ovaries in late menopausal stage subjects. Classical inflammation requires coordination among different cell types and their secretions that mediate responses against deleterious stimuli [31]. However, inflammation in ovarian tissues during aging does not present the features of classical inflammation as it is not associated with infection, widespread tissue injury, or autoimmune conditions. In contrast, age-associated inflammation in ovaries is local and may be due to metabolic imbalances [13, 32] caused by agents including hormones during aging. Aging in females is associated with the decrease in ovarian functions and cessation of synthesis of ovarian steroids including estrogen [3]. It is possible that decrease in estrogen may be

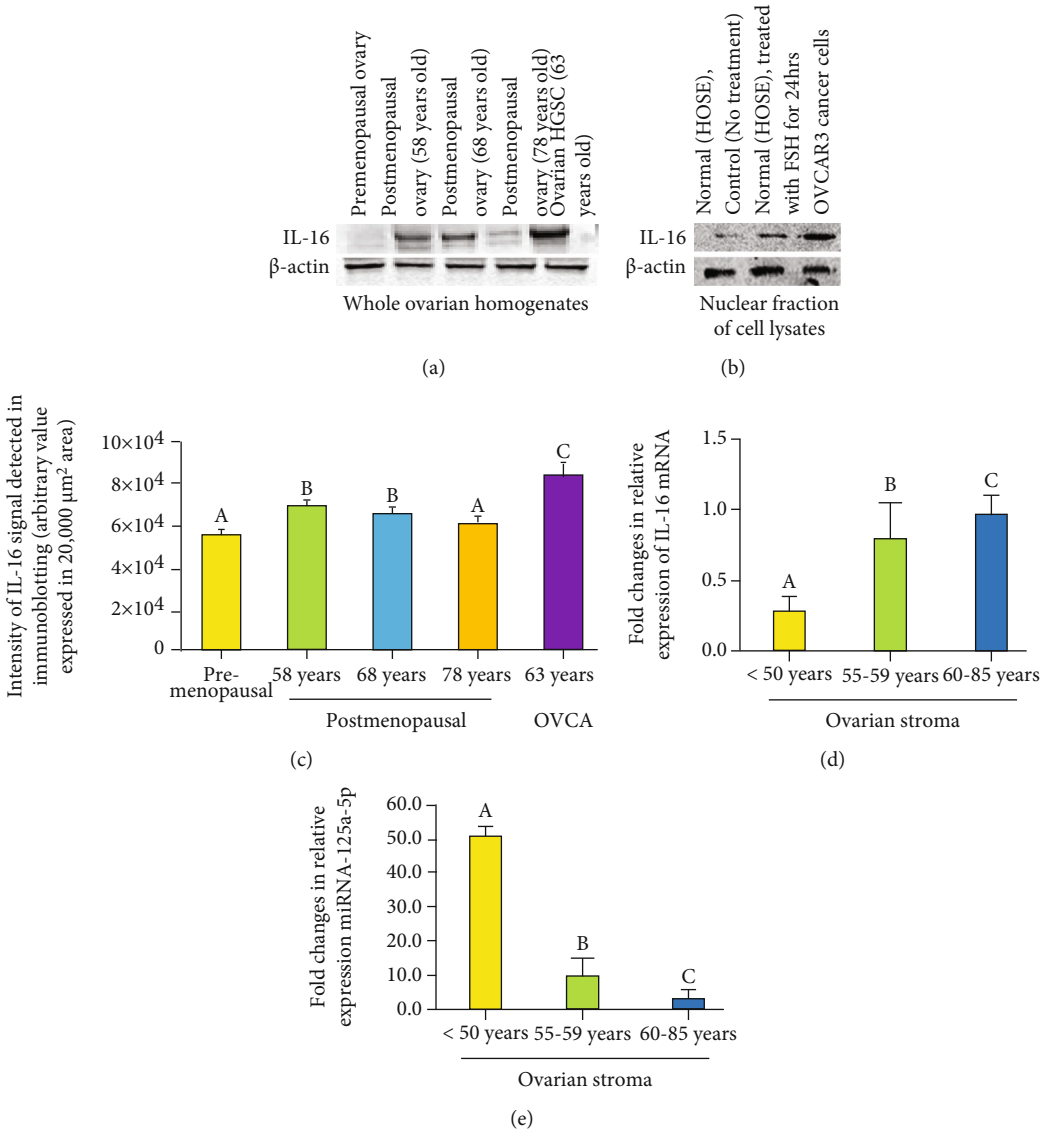

Figure 6: Changes in IL-16 protein and gene expression in the ovary during aging. (a) Western blot showing IL-16 expression during ovarian aging. (a) Changes in IL-16 protein expression in the ovary during aging. A very weak or faint immunoreactive band for IL-16 protein is seen in the ovary of a 38-year-old woman. Compared with premenopausal women, expression of IL-16 was stronger in 58- and 68-year-old postmenopausal women. However, expression of IL-16 protein was lower in 78-year-old postmenopausal ovary. As expected, expression of IL-16 protein was strongest in patient with ovarian high-grade serous carcinoma (HGSC). β-Actin protein was used as housekeeping protein. (b) Enhancement in IL-16 expression in response to exposure to follicle-stimulating hormone (FSH). Nuclear fraction in untreated (control) normal human ovarian surface epithelial (OSE) cells showed relatively weaker expression for IL-16. Compared with untreated, OSE cells treated with FSH for 24 hours showed stronger expression of IL-16. Similar patterns of expression were also observed in ovarian HGSC cells (OVCAR3). β-Actin protein was used as housekeeping protein. (c) Changes in the intensity of IL-16 expression in ovaries during aging and in ovarian tumor were detected by Western blotting. Each bar represents the mean intensity of signal for IL-16 expression (arbitrary values, reported as mean ± SEM in 20,000 μm^2 area) in three immunoblot assays. Bars with different letters are significantly different (compared to "a," "b" is significant with $P < 0.005$, compared to "b," "c" is significant with $P < 0.03$). (d, e) Changes in the relative expression of IL-16 gene and its regulator miR-125a-5p in ovaries during aging. (d) Fold changes in expression of IL-16 gene in the ovaries during aging including premenopausal and postmenopausal ovaries. Compared with premenopausal, the expression of IL-16 gene was significantly higher ($P < 0.001$) in subjects at early stage of menopause and increased further in subjects at late stage of menopause. (e) Fold changes in the expression of miR-125a-5p in the ovaries in premenopausal and postmenopausal women. Compared with premenopausal, the expression of miR-125a-5p decreased significantly ($P < 0.001$) in subjects at early stage of menopause and reduced further in subjects at late stage of menopause. Bars with different letters are significantly different. y-axis shows mean ± SEM of fold changes in IL-16 and miR-125a-5p gene expression, and bars with different letters are significantly different. Details of statistical analysis are mentioned in materials and method section of the main text.

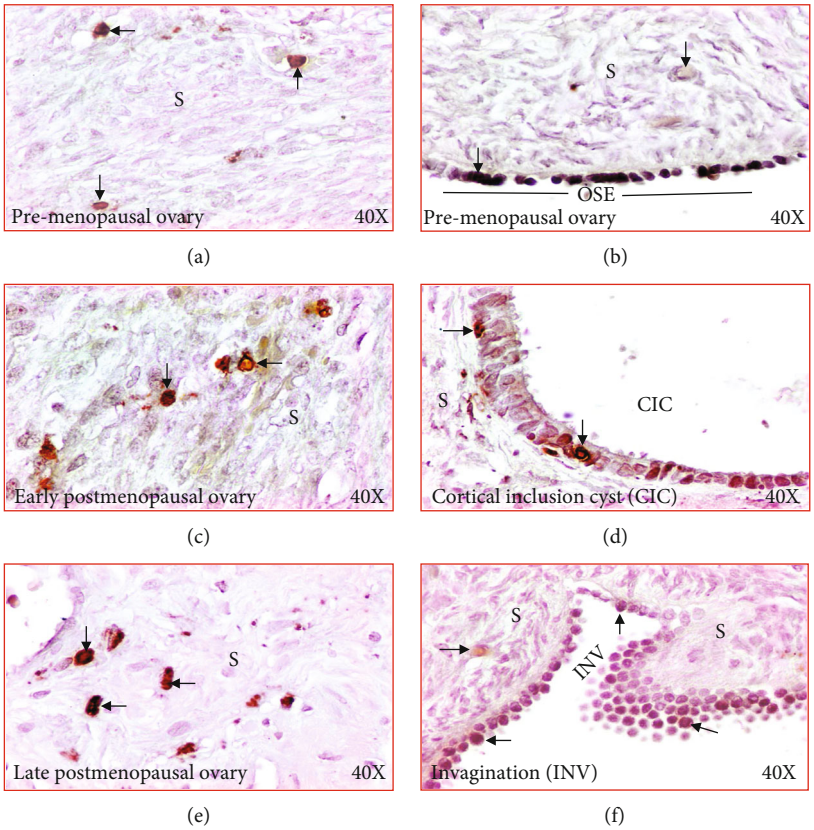

Figure 7: Changes in immunolocalization of macrophages in human ovaries during aging. (a) Section of a premenopausal ovary showing few immunopositive macrophages in ovarian stroma. (b) Section of an ovary from a subject at early stage of menopause. Compared with premenopausal, more macrophages are seen in the stroma. (c) Section of an ovary from a subject at late stage of menopause. Many macrophages are seen in the stroma. (d) Section of a premenopausal ovary showing a few macrophages in ovarian surface epithelial (OSE) layer. (e) Section of an ovary from a subject at late postmenopausal stage showing IL-16 expression by the epithelial cells (EC) in a cortical inclusion cyst (CIC) in ovarian stroma. (f) Section of an ovary from a subject at late menopausal stage showing macrophages localized in the stromal invagination (INV). S: stroma. 40×: magnifications. Arrows indicate examples of immunopositive macrophages.

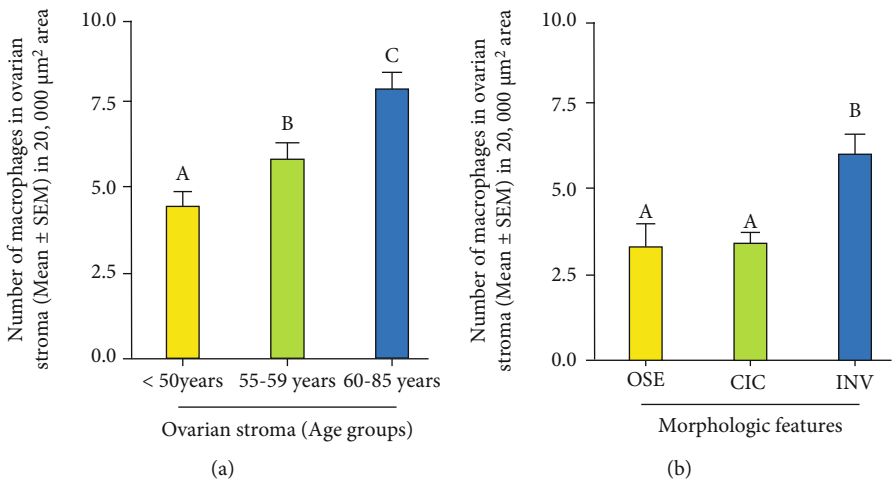

Figure 8: Changes in the frequency of macrophages in human ovaries during aging. (a) Frequency of macrophages in the ovarian stroma in premenopausal and postmenopausal ovaries. Compared with premenopausal, the frequency of macrophages was significantly higher ($P < 0.001$) in subjects at early stage of menopause (55-59 years old) and increased further in subjects at late stage of menopause (60-85 years old). (b) Population of macrophages in cortical inclusion cysts (CICs) and stromal invaginations (INVs) in menopausal ovaries. Compared with CIC, the frequency of macrophages was significantly higher in INVs ($P < 0.0001$). y-axis shows mean ± SEM of macrophages in 20,000 μm^2 area of the tissue, and bars with different letters are significantly different. Details of statistical analysis are mentioned in materials and method section of the text.

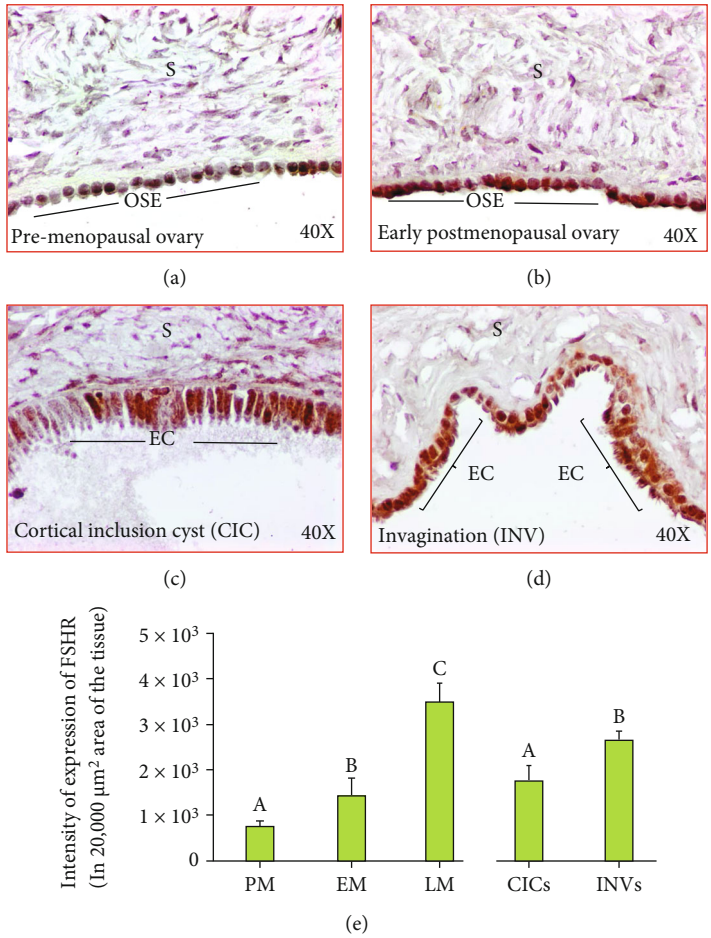

Figure 9: Expression of follicle-stimulating hormone receptor (FSHR) in ovaries during aging. (a)–(d): (a) Section of an ovary from a premenopausal subject showing a weak or moderate expression for FSHR by the ovarian surface epithelial (OSE) cells. (b) Section of an ovary from an early-stage postmenopausal subject showing strong expression for FSHR by the ovarian surface epithelial (OSE) cells. (c, d) Ovarian sections from healthy late postmenopausal women showing expression of FSHR by the epithelial cells (EC) in cortical inclusion cyst (CIC) and stromal invagination (INV), respectively. Compared with CIC, stromal INV showed stronger staining for FSHR expression. S: stroma; magnification: 40×. (e) Compared with premenopausal subjects, the intensities of FSHR expression were significantly ($P < 0.04$) higher in early menopausal women and increased further in late-stage menopausal women ($P < 0.0001$) (e). Furthermore, compared with CICs, the intensity of FSHR expression was significantly higher in INVs ($P < 0.004$) (e). y-axis shows mean $\pm$SEM ($n = 5$ for each group) in 20,000 μm^2 area of the tissue, and bars with different letters are significantly different. Details of statistical analysis are mentioned in materials and method section of the main text.

involved in the development of chronic inflammation in menopausal ovaries during aging.

Estrogen has been implicated as an anti-inflammatory agent as it has been shown to suppress the secretion of inflammatory cytokines including IL-6 and TNF-α by macrophages and dendritic cells [33]. Furthermore, circulatory levels of TNF-α, IL-1, and IL-6 have been reported to be increased in women at late menopausal stage, and their levels decreased significantly in response to hormone replacement therapy (HRT) [34]. Thus, it is possible that lack of estrogen in the ovaries in women at late menopausal stage may be a reason for the increased levels of IL-16 expression in aging ovaries. Alternatively, it is possible that persistent high levels of FSH in late menopausal stage women may be a factor for high levels of IL-16 expression.

Absence of negative feedback (due to the lack of estrogen) leads to the persistent high levels of FSH in women at late menopausal stage. Macrophages have been suggested to be a source of IL-16 synthesis [35]. Macrophage stimulating factor (MCSF) is an important cytokine which is involved in the regulation of proliferation [36], differentiation, and migration of tissue macrophages as well as is important for the maintenance of ovarian function [37]. Increasing concentrations of FSH have been shown to stimulate the expression of MCSF receptor mRNA suggesting the enhancement in MCSFR expression by FSH [38]. This action of FSH has been reported to be inhibited by estrogen treatment. Thus, it is possible that persistent high levels of FSH in postmenopausal women might be a reason of increased IL-16 production by macrophages in aging ovaries. This assumption is further supported by the results of

this study that the frequencies of macrophages were higher in women at late menopausal stage than premenopausal women. However, specific targets of FSH in aging ovaries in the context of IL-16 secretion are not fully understood.

This study showed, in addition to OSE cells, epithelial cells in CICs and INVs were positive for FSHR expression suggesting that INVs and CICs are also targets for FSH. This study further showed that compared with OSE and CICs, INVs showed stronger expression of FSHR. Thus, INVs might be a predilection site for a chronic inflammation due to persistent exposure to high FSH. INVs and CICs are features formed by the ovarian surface epithelial layer in the ovary following ovulations. This study also showed that compared to OSE cells, the frequency of IL-16-expressing cells was higher in CICs and highest in INVs. Thus, it is possible that CICs and INVs might be invaded by immune cells including macrophages (in response to ovulatory insults) which may be a source of increased expression of IL-16 in these tissues. Alternatively, chronic inflammation may be prevalent in CICs and INVs due to their persistent exposure to FSH as observed by the increased expression of FSHR in these tissues. This assumption is supported by one of the observations of this study that treatment of normal OSE cells with human recombinant FSH for 24 hours resulted in remarkable increase in nuclear expression of IL-16 with similarities in patterns of expression by OVCAR3 cancer cell lines. However, how the expression of IL-16 increases at molecular levels is not known.

MicroRNAs are endogenously synthesized short non-coding RNA molecules [39] which bind to the 3′untranslated region (UTR) of target genes and play important roles in gene regulation at the posttranscriptional level, thereby, inhibit or reduce the translation of respective target genes [40]. In this study, the levels of IL-16 expression during aging increased while the expression of its regulatory miR-125a-5p decreased significantly in women at late menopausal stage. Although specific reason(s) involved in the decrease in miR-125a-5p leading to the increase in IL-16 gene expression is not known, it is possible that changes in hormonal milieu during aging in postmenopausal women may be a factor. Because menopause is associated with the cessation of estrogen production by the ovary [41] and high persistent levels of circulating FSH, it is possible that either the lack of estrogen or high levels of FSH might have a role in the suppression of IL-16-gene regulating microRNA miR-125a-5p. Estradiol treatment has been reported to enhance expression of miR-125b [42]. Thus, it is possible that increased expression of IL-16 gene in aging ovaries might be due to the decrease in its regulatory microRNA miR-125a-5p expression because of the cessation of estrogen synthesis in postmenopausal women.

Overall, IL-16, a pro-inflammatory and chemotactic cytokine, is produced by a variety of cells including immune cells and epithelial cells of different organs [43–45]. IL-16 has been implicated in several cancers including OVCA [26, 46]. OVCA in most cases is a malignancy of postmenopausal women and the median age of OVCA incidence is 63 years. Longstanding unresolved oxidative stress and chronic inflammation have been suggested as predisposing factors for malignancy including OVCA [13, 14]. Stromal INVs and CICs are structures formed by the ovarian surface layer following ovulation and have been shown to be a predilection site for malignant transformation [47]. Increased expression of IL-16 in these structures as observed in this study suggests the prevalence of chronic inflammation in these structures. Moreover, deletions in the chromosome 19 with approximately 60% loss of heterozygosity have been reported to be associated with OVCA [48, 49], and interestingly, miR-125a-5p is localized in the 19q13.41 locus of this chromosome. The consequence of increase in nuclear expression of IL-16 due to FSH exposure is not known. It is possible that, following enhancement in expression as a result of chronic oxidative stress due to the persistent exposure to FSH, IL-16 may translocate to the nucleus and lead to the formation of mutagenic DNA adducts. Previous reports suggest the formation of mutagenic DNA-adducts due to oxidation of DNA bases which may lead to malignancy [50]. Thus, dysregulation in miR-125a-5p during aging may be a reason for increased expression of IL-16 in postmenopausal women and may also increase the risk of developing OVCA as it is a disease of postmenopausal women.

In conclusion, results of this study suggest that expression of IL-16, a proinflammatory and chemotactic cytokine, increases during aging in the ovaries in postmenopausal women. This increase in IL-16 expression was localized in CICs and INVs, the two structures in the ovary formed following ovulation and are sites with risk of malignant transformation. Moreover, increase in IL-16 expression was associated with its regulator microRNA miR-125a-5p, also a tumor suppressor microRNA. Thus, chronic inflammation in the ovaries in postmenopausal women may predispose them to ovarian pathology including malignant transformation in ovaries.

Data Availability

All data supporting the conclusions of this article are included in the article.

Conflicts of Interest

The authors have no conflict to declare

Authors' Contributions

J.R. performed all experiments, curated the data and their analysis, and drafted the manuscript. P.B. reviewed and edited the manuscript. S.B. helped in formal data analysis, review, and editing of manuscript. A.B. conceptualized the study, acquired the resources, supervised the study, reviewed, edited, and finalized the manuscript.

Acknowledgments

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Supplementary Materials

Figure S-1: Control (negative) staining for antibodies used in the present study. Figure S-2: Intensity of β-actin protein expression in healthy ovarian tissues and ovarian high grade serous carcinoma. Figure S-3: (a) Intensity signal of IL-16 expression in the nuclear fraction of untreated normal OSE cells, the nuclear fraction of OSE cells treated with FSH for 24 hours, and OVCAR3 cells. (b) Intensity of β-actin protein expression in the nuclear fraction of normal OSE cells untreated or treated with FSH for 24 hours, and OVCAR3 cells. *(Supplementary Materials)*

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Review Article

Oxidative Stress and 4-hydroxy-2-nonenal (4-HNE): Implications in the Pathogenesis and Treatment of Aging-related Diseases

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Oxidative stress plays an important role in the development of aging-related diseases by accelerating the lipid peroxidation of polyunsaturated fatty acids in the cell membrane, resulting in the production of aldehydes, such as malondialdehyde and 4-hydroxy-2-nonenal (4-HNE) and other toxic substances. The compound 4-HNE forms adducts with DNA or proteins, disrupting many cell signaling pathways including the regulation of apoptosis signal transduction pathways. The binding of proteins to 4-HNE (4-HNE-protein) acts as an important marker of lipid peroxidation, and its increasing concentration in brain tissues and fluids because of aging, ultimately gives rise to some hallmark disorders, such as neurodegenerative diseases (Alzheimer's and Parkinson's diseases), ophthalmic diseases (dry eye, macular degeneration), hearing loss, and cancer. This review aims to describe the physiological origin of 4-HNE, elucidate its toxicity in aging-related diseases, and discuss the detoxifying effect of aldehyde dehydrogenase and glutathione in 4-HNE-driven aging-related diseases.

1. Introduction

Aging is a natural phenomenon that all humans undergo, and delaying this process is a major challenge faced by the scientific community. Based on the research on aging mechanisms, mitochondria-derived free radicals are considered to play an important role in the process of cell senescence and the development of aging-related diseases. These mitochondria-derived free radicals include reactive oxygen species (ROS) [1], such as superoxide radicals ($O_2^{\bullet-}$), H_2O_2, and OH^-, as well as several reactive nitrogen species (RNS) [2]. An *in vivo* imbalanced ratio of antioxidant enzymes to ROS/RNS triggers an oxidative stress condition that consequently leads to oxidative protein modification [3], DNA damage due to altered methylation processes [4], and lipid peroxidation (LPO) of the cell membrane, thereby affecting cell proliferation, differentiation, apoptosis, and its related signal pathways. LPO of cellular membranes produces many secondary products, such as aldehydes and other toxic substances. Aldehydes, among which 4-HNE is the most toxic form, can accelerate the damage caused by oxidative stress. This mechanism of toxicity is primarily

attributed to the binding of 4-HNE to protein, which inactivates some antioxidant enzymes and further aggravates oxidative stress, and ultimately apoptosis. Moreover, in a state of oxidative stress, cholesterol is another lipid compound that is susceptible to ROS attack which gives rise to an extensive series of molecules, termed oxysterols (7-ketocholesterol, 7β-hydroxy-cholesterol, 5α,6α- and 5β,6β-epoxy-cholesterol, and cholestan-3β,5α,6β-triol) [5, 6]. These damaging compounds can trigger the development of senile and neurodegenerative diseases [7] (such as Alzheimer's disease [AD], Parkinson's disease [PD], Huntington's disease [HD], and amyotrophic lateral sclerosis [ALS]), immune diseases, tumors [8], hearing loss [9], cardiovascular diseases (such as coronary heart disease [10]), and ophthalmic diseases (such as macular degeneration [11] and dry eye [12]).

With the recent technological advances such as the omics approach for redox proteins and detection of ROS-modified protein targets, clinical researchers can now better understand the mechanism of oxidative stress and aging-related diseases in the human body [13, 14]. Furthermore, improved 4-HNE-targeted detection technology and the application of multifunctional naturally occurring antioxidants have provided new research impetus for the treatment of 4-HNE-related oxidative stress diseases [15]. To date, antioxidants targeting the scavenging oxygen-free radicals have been identified to prevent the harmful effects due to oxidative stress. Aldehyde dehydrogenase (ALDH) and glutathione (GSH) have shown significant antioxidant effects on age-related diseases caused by ROS; hence, it is necessary to comprehensively explore the therapeutic potential of ALDH and GSH through in-depth experimental and clinical research [16–18]. The purpose of this review was to explain the physiological origin of 4-HNE, summarize its toxicity effects in aging-related diseases, and highlight the detoxification effects of GSH and ALDH.

2. Lipid peroxidation

In addition to ROS, lipids can be oxidized by enzymes like lipoxygenases, cyclooxygenases, and cytochrome P450. In particular, ROS are highly reactive and can rapidly react with lipids, proteins, and nucleotides. The accumulation of cellular ROS under oxidative stress conditions results in the induction of LPO and glycoxidation reactions, leading to the elevated endogenous production of reactive aldehydes and their derivatives such as glyoxal, methylglyoxal (MG), malondialdehyde (MDA), and 4-hydroxy-2-nonenal (4-HNE), which ultimately lead to advanced lipoxidation and glycation end products (ALEs and AGEs, respectively [19, 20]. MDA is the most mutagenic byproduct of LPO, whereas 4-HNE is the most toxic. Membrane phospholipids containing a large number of polyunsaturated fatty acids (PUFAs) are easily attacked by ROS and undergo LPO due to oxidative stress. Moreover, elevated intracellular iron concentration and a depletion of antioxidant GSH both increase ROS levels, consequently leading to ferroptosis, a form of cell death due to overwhelming membrane LPO [21–23]. LPO reactions in biofilms mainly occur through enzymatic and nonenzymatic pathways, whereby the nonenzymatic pathway is divided into two mechanisms: LPO mediated by either non-free radicals or free radicals [21]. In recent years, free radical-mediated nonenzymatic LPO has become the main focus of research on aging-related diseases because it leads to the formation of many secondary products, such as aldehydes, malondialdehyde, 4-HNE, and acrolein [24]. Because of their high reactivity and toxicity to the biological components of cells, these substances have attracted significant attention. Among them, 4-HNE is the most extensively researched, is frequently used as an oxidative stress marker [25, 26], can be used with oxysterols to assess oxidative stress [27, 28], and has been associated with the pathogenesis of cancer [29, 30], neurodegenerative diseases [31], diabetes [32], and other diseases.

3. 4-Hydroxy-2-nonenal

3.1. Sources of 4-HNE. The aldehyde compound 4-HNE is highly reactive and forms adducts with cellular proteins and DNA. It is also an effective signal molecule that regulates the mitogen-activated protein kinase (MAPK) pathway and the activity of redox-sensitive transcription factors (nuclear factor erythroid 2-related factor 2 [Nrf2], activating protein-1 [AP1], and NFκB) [33], and influences several key signaling pathways, including the MAPK pathway, Jnk, and p38, PKC-β and δ, and Nrf2 [34].

A dyshomeostasis in ROS leads to the oxidative stress reaction of PUFAs, which can be divided into nonenzymatic and enzymatic pathways, as shown in Figure 1. In the nonenzymatic pathway, after a series of reactions, PUFAs produce lipid hydroperoxide (LOOH), 15-hydroperoxyeicosatetraenoic acid (15-HpETE), or 13-hydroperoxy-linoleic acid (13-HpODE), followed by the participation of transition metals, Hock rearrangement, and C-C $\rightarrow$ C-H migration. Then, alkanaldehyde, alkenaldehyde, and γ-alkenaldehyde are formed after fracture, and finally, 4-HNE is produced [34]. In the enzymatic pathway, according to the oxidized fatty acid (i.e., its length, number, and position of double bonds), the oxidation products are different in length, unsaturation, and substitution number, resulting in different secondary products. Fatty acids can be divided into two types according to the position of the first double bond from the methyl end: ω-3 and ω-6 fatty acids, which affect the possible position of cleavage and the corresponding cleavage products. The compound 4-HNE is mainly produced by the precursor substances, ω-6PUFAs (linolenic acid, γ-linolenic acid, and arachidonic acid) through 15-lipoxygenase (15-LOX) [35]. ω-3-PUFAs, including alpha-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid, can also be cleaved to produce many reactive aldehydes. Moreover, arachidonic acid can be oxidized by cyclooxygenase-2(COX-2) to reactive carbonyl compounds such as 4-oxo-2-nonenal, 4-hydroperoxy-2-nonenal, 4-hydroxy-2E, 6Z-dodecadienal, and trans-4-HNE [36]. Among these secondary products of aldehydes, 4-HNE is undoubtedly the most studied and is considered a useful biomarker for LPO [37].

3.2. Characteristics of 4-HNE. 4-HNE is considered one of the major mediators of oxidative stress in cells and tissues, that collectively lead to cell senescence by affecting the expression of various senescence-related signaling pathways

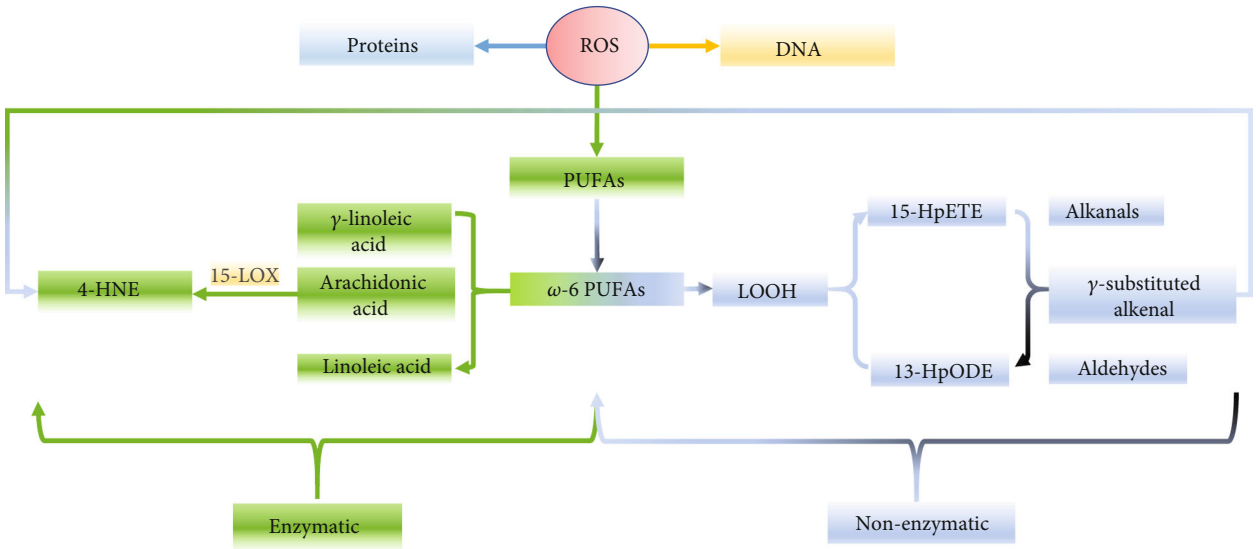

Figure 1: Production pathway of 4-hydroxy-2-nonenal (4-HNE). 4-HNE is produced by non-enzymatic and enzymatic pathways in ω-6 PUFAs induced by ROS.

[38] (such as NF-κB, Nrf2, Akt/PKB, and mTOR), as shown in Figure 2. The combination of the two conjugated functional groups of 4-HNE, the carbonyl group (-C=0), and a double bond (C2/C3, -C=C-) facilitates the reaction of 4-HNE with biological molecules (including lipids, nucleic acids, and proteins) [39]. A mass spectrometry-based proteomic analysis of oxidative stress found that the electrophilic double bond and nucleophilic amino acid residues on proteins enable 4-HNE to form Michael adducts with lysine (Lys) [40], histidine (His) [41], and cysteine (Cys) [42] residues to increase the molecular masses of these amino acids by 156 Da (the molecular mass of HNE), or form Schiff-base adducts with its aldehyde group and Lys to an increase in the mass by 138 Da (Schiff-base formation with a net loss of water) [43, 44]. Moreover, the research of proteomic analysis of *in vitro* histone carbonylation sites showed that Schiff-base modification is labile and reversible, while that of Michael adducts are stable and non-reversible [45]. The Michael adducts can remain in cells for several hours before degradation, leading to the dysfunction of the targeted biomolecules. Therefore, the detection of HNE-biomolecular adducts is considered a valuable tool for evaluating various pathophysiological conditions associated with aging-related diseases.

4. Oxidative Stress, ALDH, and GSH

The human genome contains 19 known functional aldehyde dehydrogenase (ALDH) genes that are required for the biosynthesis of retinoic acid (RA) and other functional cellular or molecular regulators [46]. Previous mouse transplantation studies have demonstrated the role of ALDH as a molecular marker for hematopoietic stem cells (HSCs) and leukemic stem cells (LSCs). ALDH2 is the most active ALDH consisting of 517 amino acids to form 456 kDa subunits, each containing three domains. Recent studies have shown that ALDH2 plays a role as a stem cell marker and regulates cell functions related to self-renewal, expansion, differentiation, and resistance to drugs and radiation [47]. Moreover, ALDH is a key enzyme for the detoxification of endogenous and exogenous aldehyde substrates through $NAD(P)^+$ dependent oxidation [48]. ALDH2 plays a role in the oxidative metabolism of toxic aldehydes in the brain, such as catecholaminergic metabolites (DOPAL and DOPEGAL) and 4-HNE [49].

Glutathione (GSH) is one of the most abundant low-molecular-weight mercaptans synthesized in cells. It is synthesized by adding Cys to glutamic acid and glycine. It plays a vital role in protecting cells from oxidative damage and toxicity of xenophilic reagents, as well as in the maintenance of redox homeostasis [50]. Lipids are one of the main substrates of glutathione-related protein (GSTM2), which can induce young cells to combine with 4-HNE, thereby reducing the content of 4-HNE in the liver, kidney, and serum of old mice [51]. Therefore, GSH plays a role in preventing aging-related diseases.

5. Physiological Role and Pathological Implications of Oxidative Stress derived 4-HNE

Oxidative stress leads to the production of LPO, which is one of the main sources of free radical-mediated damage that leads to the production of many secondary aldehydes, such as 4-HNE, 4-hydroxydodeca-(2E, 6Z)-dietary (4-HDDE), and 4-hydroxy-2E-hexenal (4-HHE) [52]. The mammalian brain is a highly oxidized organ [53], and PUFAs and relatively weak antioxidant defenses [54] render the brain vulnerable to free radical oxidative stress. Aging brain often has enhanced free radicals and decreased bioenergy [55]. Studies have shown that oxidative damage mediated by free radicals is closely related to the pathogenesis of aging-related neurodegenerative diseases [56–58]. The compound 4-HNE

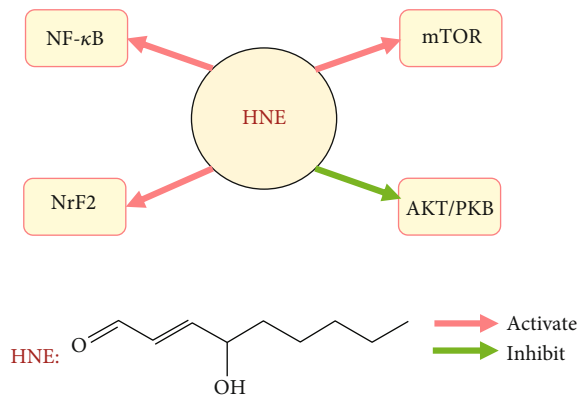

Figure 2: Summary of 4-hydroxy-2-nonenal (4-HNE) involvement in an aging-related signaling pathway. 4-HNE leading to NF-κB, Nrf2, and mTOR pathway activation, and inhibiting AKT/PKB pathway activity.

and the 4-HNE-protein complex are often detected in patients diagnosed with neurodegenerative diseases. Studies have shown that patients with AD, PD, HD, or ALS have increased levels of 4-HNE-protein adducts in their body fluid [59–61]. The 4-HNE-protein adducts can induce autoimmunity, and may be associated with the development of neurodegenerative diseases [62, 63]. Wang et al. [64] found a significant increase in anti-4-HNE-protein adduct antibody levels in $MRL^{+/+}$ mice treated with trichloroethylene.

5.1. Oxidative Stress-derived 4-HNE and Alzheimer's disease (AD). AD is a neurodegenerative disease with heterogeneous clinical symptoms, usually manifested as language, visuospatial, and executive dysfunctions [65]. Previous studies have suggested that AD is mainly caused by the amyloid plaques of amyloid-β (Aβ). However, all strategies and compounds for inhibiting Aβ deposition have failed in clinical trials [66], and the area with the highest deposition of Aβ does not coincide with the area with formation of neurofibrillary tangles (NFTs) and acute loss of synapses and neurons. Therefore, current focus of research has shifted to the hyperphosphorylated tau protein, which is affected by many pathological factors (such as abnormal activation of kinase, abnormal gene expression, and chronic stress), which is clinically known to cause excessive aggregation of NFTs [67]. There is also evidence that the brain tissue of AD patients is affected by toxic substances such as peroxides, alcohol, aldehydes, free carbonyls, and ketones produced by LPO due to oxidative stress. After the Aβ peptide is inserted into the neuronal lipid bilayer, it undergoes peroxidation to produce 4-HNE, which then covalently binds to the key neuronal membrane and cytosolic and mitochondrial proteins through a Michael addition reaction, leading to functional disorders of key neuronal proteins, neuronal death, and loss of cognitive ability [68].

The main neuronal protein binding sites for HNE are Lys, His, and Cys residues [69]. ApoE expression regulation and ApoE gene polymorphism play important roles in neurodegenerative diseases such as AD and PD, and other diseases [70]. The protein apoE2 has two Cys residues that can bind to 4-HNE, which can prevent neuronal protein damage. However, both Cys residues of ApoE4 could be replaced by arginine (Arg) residues, resulting in no 4-HNE binding to neuron proteins and leading to a cascade of events such as Aβ fibril deposition, Aβ oligomer production, neurofibril tangle formation, neuronal death, decreased synaptic plasticity associated with learning and memory, asymmetric lipid bilayer composition, loss of lipid homeostasis, and oxidative stress, which collectively increases the risk of AD [71, 72].

5.1.1. ALDH and AD. A previous study [73] showed that accumulation of 4-HNE adducts, Aβ protein, and p-tau protein increased significantly in the hippocampus of $ALDH2^{-/-}$ mice, indicating that ALDH2 significantly affected the AD process in mice. Joshi et al. [74] found that aldehyde dehydrogenase-2 deficiency (ALDH2∗2) reduced the clearance of toxic aldehydes, resulting in additional ROS production, followed by aldehyde addition to mitochondrial proteins, leading to mitochondrial dysfunction, ATP depletion, and ROS accumulation. These pathological changes in neurons and astrocytes lead to neuroinflammation, which affects the ability of glial cells to remove cell fragments. Therefore, activating ALDH2∗2 and increasing ALDH2-related compounds, such as Alda-1 (an activator of ALDH2 and ALDH2∗2), could be a potential therapeutic approach to slow down AD pathogenesis.

5.1.2. GSH and AD. As an important cellular antioxidant, GSH balances the production and binding of free radicals and protects neurons from oxidative damage. It was observed that the level of GSH and the proportion of GSH/GSSG decreased in $APP^{NL\text{-}GF/NL\text{-}GF}$ mice. After oral administration of GSH3, GSH levels increased in a dose-dependent manner and restored the ratio of GSH/GSSH. Concurrently, the level of 4-HNE in the mouse hippocampus decreased significantly [75], indicating that GSH can arrest the pathogenesis of AD by eliminating the toxic aldehyde, 4-HNE.

5.2. Oxidative Stress-derived 4-HNE and Parkinson's disease (PD). PD is a major neurodegenerative disease. It is characterized by motor symptoms such as tremor, stiffness, muscular rigidity, and postural instability; and a series of non-motor symptoms, including autonomic and cognitive dysfunction [76]. The hallmark pathological features of PD are loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) [77] and abnormal amyloid accumulation of misfolded α-synaptic protein (α-syn) in the formation of inclusions called Lewy bodies [78]. Studies have shown that the causes of dopaminergic neuronal death include impaired mitochondrial function [79] and dysbiosis of gut microbiota (imbalanced intestinal flora) [80].

Evidence has shown that high levels of oxidative stress in the SNc region of normal brain could be the triggering factor for a series of biochemical changes that lead to the death of dopaminergic cells. The early compensatory changes in dopamine caused by the degeneration of substantia nigra cells was found to increase oxidative stress and 4-HNE accumulation [81]. 4-HNE may affect PD in two main pathways.

First, the complex formed following covalent modification of α-syn by 4-HNE (4-HNE/α-syn) tends to form more oligomers and fibrils than the simple α-syn, subsequently forming amyloid fibers that increase the toxicity to dopaminergic neurons [61, 82]. Zhang et al. [83] found that in neurons exposed to 4-HNE, the immunoreactivity of α-syn aggregates in whole neurites and cell bodies of different sizes, indicating that 4-HNE can induce the aggregation of α-syn in primary cortical neurons of rats.

The second pathway involves the destruction of the ubiquitin-proteasome system (UPS) by 4-HNE. UPS participates in the degradation of "bad" proteins related to damage, death, and modification by oxidation products such as 4-HNE [84]. However, the chemical structure, stability, and function of ubiquitin may be damaged by 4-HNE modification, thereby increasing cytotoxicity and affecting the process of PD. It was reported that 4-HNE can bind to the 26S proteasome [85], lead to the non-degradation of "bad" proteins and the accumulation of 4-HNE-protein complexes, hence affecting the pathology of PD. Additionally, some studies have shown that in addition to the direct relationship between 4-HNE/α-syn and dopaminergic transmission changes, 4-HNE can directly affect dopamine transmission by acting on dopamine receptors [86] and promotes the pathogenesis of PD.

5.2.1. ALDH and PD. To verify the role of 4-HNE and other biogenic aldehydes in impaired detoxification homeostasis that leads to the pathogenesis of PD, Wey et al. [87] established an Aldh1a1−/− × Aldh2−/− mice PD model. The results of gait analysis and the accelerated rotation rod test indicated age-dependent motor performance defects: a significant decrease in the number of neurons immunoreactive to tyrosine hydroxylase (TH) in the substantia nigra, a decrease in dopamine and metabolites in the striatum, and an increase in neurotoxic bioaldehydes such as 4-HNE. These results support the hypothesis that damage to biological aldehyde detoxification mechanisms affects the pathophysiology of PD. Another study [88] determined the role of aldehyde dehydrogenase 1A1 (ALDH1A1) in mediating 4-HNE toxicity in PC12 cells using excessive ALDH1A1 and ALDH inhibitor disulfiram. Results showed that ALDH1A1 was downregulated in the brain tissue of patients with PD, 4-HNE toxicity was elevated, and ALDH activity was negatively correlated with the content of 4-HNE-protein adducts. Therefore, it is considered that the ALDH1A1 activator has a neuroprotective effect on patients with PD by reducing the content of 4-HNE-protein and inhibiting cytotoxicity [89, 90].

5.2.2. Glutathione (GSH) and PD. Brain GSH levels in patients with PD are decreased, and the dopamine D2 receptor (DRD2) in astrocytes can regulate the synthesis of GSH through pyruvate kinase isozyme type M2 (PKM2)-mediated transactivation of Nrf2. In addition, pyridoxine can dimerize PKM2 to promote GSH biosynthesis [91]. Further experiments showed that pyridoxine supplementation increased the resistance of dopaminergic neurons in the substantia nigra to neurotoxicity in wild-type mice and astrocyte DRD2 conditional knockout mice. Thus, PKM2 may be a potential target for PD treatment.

5.3. Oxidative Stress derived 4-HNE and Cancer. ROS are involved in cancer-related cellular processes, such as proliferation, apoptosis, differentiation, cell migration, and DNA damage [92, 93]. However, due to their short half-lives, the carcinogenic effects of ROS are limited to the areas adjacent to their production. Nevertheless, the secondary product of LPO, 4-HNE is known to exert more complex carcinogenic effect on cell activity.

The carbonyl group on C1, the double bond carbon at the C3-C4 site, and the electron-absorbing hydroxyl group on C4 in 4-HNE further aggravate the electrophilicity of C3, which makes it easy for C3 to covalently bind to the primary amine of amino acid residues to form a Schiff-base modification [45]. The Schiff base produced by the covalent binding of 4-HNE-Lys forms a very stable pyrrole compound after cyclization. Pyrrole and its derivatives exhibit significant anticancer activities [94]. Therefore, 4-HNE can spread from the site of origin, change the structure and function of a corresponding protein, and lead to carcinogenesis. DNA damage plays an important role in mutagenesis, carcinogenesis, aging, and other pathophysiological conditions. 4-HNE can covalently bind to DNA, affecting the genomic function of normal cells, leading to carcinogenesis [95]. In addition to its direct cytotoxicity, 4-HNE is involved in regulating cellular signaling pathways, especially the Nrf2/Keap1/ARE pathways [96, 97]. Studies have shown that the distribution of 4-HNE in squamous cell carcinoma depends on the clinical stage and histological grade of these tumors [98]. Immunohistochemical analysis showed that the expression levels of 4-HNE in well, moderately, and poorly differentiated prostate cancer (PCa) were significantly higher than those in benign prostatic hyperplasia (BPH) tissue [99]. Therefore, the expression of 4-HNE is related to the grade of prostate cancer, making it a potential new biological reference marker for the prognosis of prostate cancer.

4-HNE plays a contrasting role in promoting cancer development and arresting tumor growth via upregulating the metabolic pathway of RLIP76 detoxification in tumors [96]. Low levels of 4-HNE can increase the differentiation markers of breast cancer stem cells (BCSCs). In contrast, high levels of chronic 4-HNE increase the concentration of GSH and Nrf2, hence increasing antioxidant protection [100].

5.3.1. ALDH and Cancer. ALDH is considered a reliable marker of cancer stem cells (CSCs), which is widely used to enrich CSC subsets from various cell lines and solid tumors [101]. ALDH1 has three main isotypes, ALDH1A1, ALDH1A2, and ALDH1A3, which are involved in self-renewal, differentiation, and self-protection, and are markers of normal tissue stem cells (SCs) and cancer stem cells (CSCs) [48]. ALDH2 is a key enzyme that protects the heart from oxidative stress by consuming 4-HNE, and the metabolism of ROS and 4-HNE is thought to be deeply involved in cancer cell death. Hence, ALDH2 is considered to play an important role in cancer treatment [102].

5.3.2. GSH and Cancer. The imbalanced redox homeostasis leads to an increased ROS content in tumor cells and cell death, which is an effective cancer treatment strategy. Studies have shown that GSH depletion and biosynthesis inhibition can reduce the concentration of GSH in cancer cells and enhance the therapeutic effect of photodynamic therapy (PDT) in cancer patients [103]. Moreover, the increased expression of GSH can protect cells from 4-HNE-induced cell damage and reduce cancer risk. It was reported that homocysteine (Hcy) induced the expression of Nrf2 protein and increased the expression of glutathione in HepG2 cells in a concentration-dependent manner, indicating that Hcy can induce GSH expression and mediate the antioxidant transcription factor Nrf2 to protect HepG2 cells from damage induced by the LPO secondary product, 4-HNE [104].

5.4. Oxidative Stress-derived 4-HNE and aging-related hearing loss (ARHL). ARHL results from various factors, including aging, noise, ototoxic chemicals, heredity, epigenetic variables, and lifestyle. ROS accumulation and oxidative damage lead to abnormal cell function, damaged cell vitality, and eventually lead to functional decline and aging of the auditory system [105]. The compound 4-HNE is one of the most abundant end-products of LPO, which can lead to ARHL. A study showed that 4-HNE levels in aged deaf mice were higher than those in the young control group [106]. This is related to the decrease in spiral ganglion neuron (SGN) density and the thickness of hair cells and vascular stria in the cochlea of aged mice. Additionally, sublethal concentrations of H_2O_2 induced ROS, which then led to DNA damage. Following exposure to H_2O_2, mitotic cochlear implant cells showed the key characteristics of senescent cells, including significantly increased expression of p21, p38, and p-p38, decreased expression of p19 and BubR1, and positive labeling of β-galactosidase. It was suggested that the DNA damage response induced by ROS drives the senescence of cochlear cells and promotes the pathology of ARHL [107].

5.4.1. GSH and ARHL. Glutathione transferase (GST), an important detoxifying enzyme, protects cells by catalyzing the binding of toxic compounds to reduced GSH. GSTA4 has a high catalytic effect on 4-HNE, and the combination of GSTA4 and GSH is considered the most effective means to eliminate 4-HNE. GSTA1 and GSTA2 can catalyze the reduction of fatty acid hydrogen peroxide (FA-OOH) and phospholipid hydrogen peroxide (PL-OOH), which are then reduced to the corresponding alcohol oxidized glutathione (GSSG) and water as by-products, thus preventing the formation of 4-HNE. GSTA4 and GSTA5 combine 4-HNE with GSH to form a GSH-4-HNE conjugate (GSH-4-HNE), which is then eliminated by the transmembrane transporters [9].

Cisplatin treatment was used to increase the level of 4-HNE in SGNs of WT female mice and the activity of GSTA4 on 4-HNE in the cochlea. In female $GSTA4^{-/-}$ mice, cisplatin treatment increased the content of 4-HNE in cochlear neurons. In CBA/CaJ mice, ovariectomy decreased the mRNA expression of Gsta4 and the GSTA4 protein levels in the inner ear. Therefore, GSTA4-dependent detoxification may play a role in estrogen-mediated neuroprotection [108]. Moreover, GSTA4-mediated 4-HNE detoxification may play a role in protecting cochlear cell death, noise exposure, and age-related hearing loss [106].

5.5. Oxidative Stress-derived 4-HNE, aging-related macular degeneration (AMD), and dry eye. AMD is a disease that affects the macular region of the retina and can lead to a gradual loss of central vision. AMD is a multifactorial disease and its pathogenesis manifested as various disorders of the complement system and lipids, angiogenesis, inflammation, and extracellular matrix pathways [109]. Oxidative stress-induced retinal pigment epithelium (RPE) damage is considered a key factor in AMD pathology. Continuous exposure to oxidative stress in RPE cells can lead to the accumulation of damaged cellular proteins, lipids, nucleic acids, and organelles, including mitochondria [110], which aggravates AMD symptoms. RPE is rich in lipids, has high metabolic needs, is prone to LPO that accumulates 4-HNE, which made 4-HNE an ideal AMD retinal biomarker. Additionally, 4-HNE can induce apoptosis, lysosome imbalance, and lipofuscin production in RPE by activating a variety of molecules, such as NF-κB, p53, Caspase-3, and NOX4, thus destroying the self-repair function of photoreceptor cells and causing AMD [111].

Oxidative stress and subsequent chronic inflammatory mediators can lead to the death of RPE cells, which is a therapeutic target for AMD. However, the molecular mechanism underlying the link between oxidative stress and inflammation remains unclear. A cytokine array was used to evaluate cytokine production in RPE induced by 4-HNE [112]. Molecular analysis confirmed that 4-HNE induced the production of IL-6, IL-1, and TNF-α by promoting the extracellular outflow of HSP70 and induced the production of low concentrations of IL-10 and TGF-β, thus playing a pro-inflammatory role in RPE cells.

Epidemiological studies have shown that the incidence of xerophthalmia increases with age, indicating an association with aging. In a dry eye model caused by continuous exposure to low humidity airflow for 30 days, the immunoreactive immune stress marker 4-HNE was found to increase with increased fluorescence, a clinical feature of epithelial lesions. In patients with xerophthalmia, it was found that the expression of the lipid peroxide marker 4-HNE on the surface membranes of eye increased compared to patients without xerophthalmia [113], suggesting that xerophthalmia is associated with an increased level of 4-HNE, a secondary product of oxidative stress.

5.5.1. ALDH and AMD. A new model of RPE degeneration *in vivo* was established using spermidine as an inducer. Spermidine (20–30 nmol/eye) could destroy retinal electrophysiology and barrier function, causing degeneration of the retinal pigment epithelium and photoreceptors. On the 7th day after using ALDH (1.5 U/eye), the increase in permeability of blood-retinal barrier (BRB) induced by spermidine and the degeneration of RPE and photoreceptors were significantly inhibited. In addition, an acrolein-modified

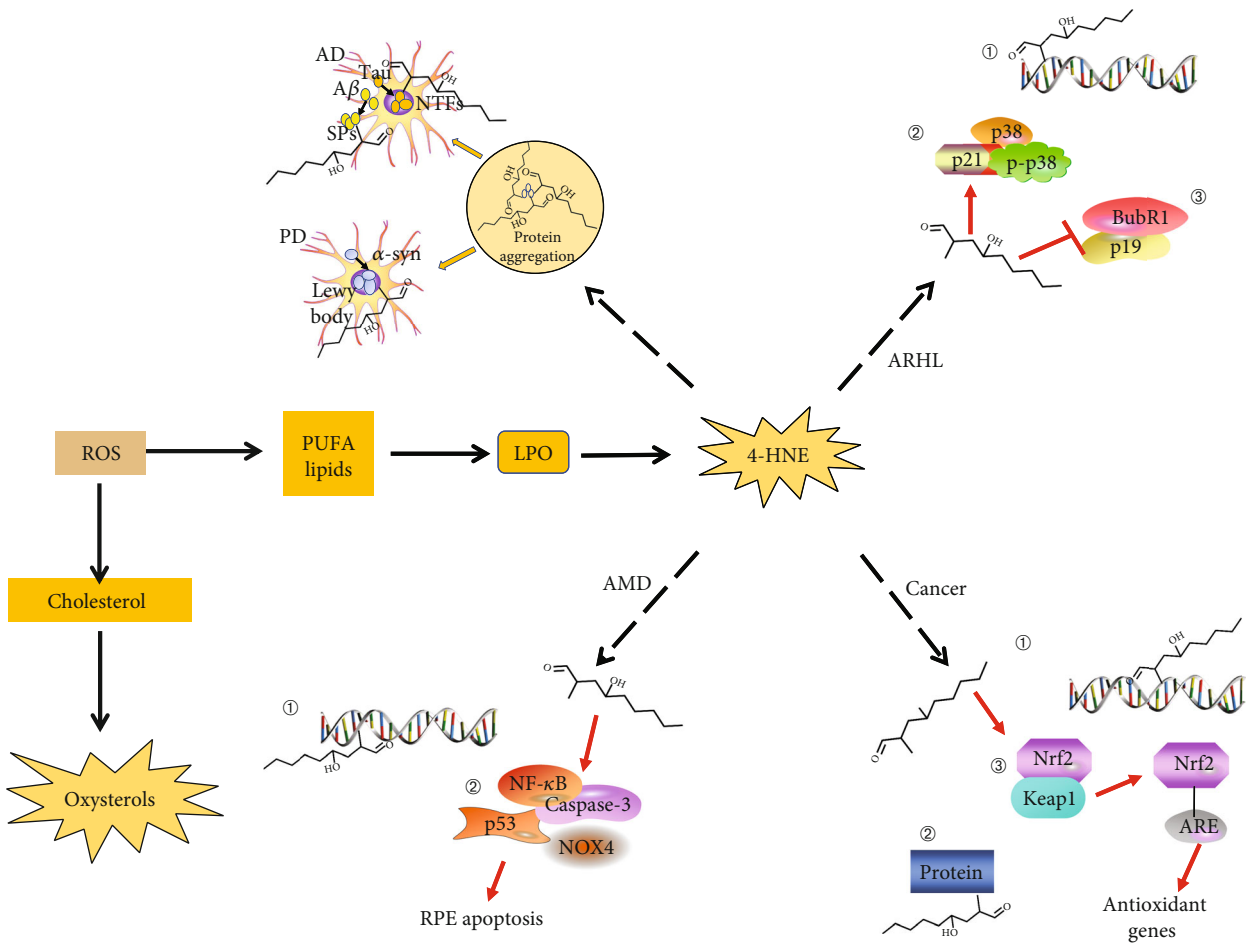

Figure 3: Role of 4-HNE in aging-related diseases. AD and PD: 4-HNE induces protein aggregation, abnormal aggregates of Aβ forms SP, and hyperphosphorylation of tau protein to form NFTs, which eventually leads to AD; aggregates of α-synuclein form Lewy body-like inclusions leading to PD. ARHL: 4-HNE (1) causes DNA damage; (2) increases p21, p38, and p-p38 activity; (3) reduces p19 and BubR1 activity. Cancer: 4-HNE (1) causes DNA damage; (2) affects protein function; (3) directly modifies cysteine residues (Cys 513, 518) on KEAP1, thus leading to KEAP1/Nrf2 pathway activation and increases the expression levels of Nrf2-ARE target genes, thus, activating the antioxidant-related pathways to influence cancer progression. AMD: 4-HNE (1) causes DNA damage; (2) activates NF-κB, p53, Caspase-3, and NOX4 activity, thereby, inducing retinal pigment epithelium (RPE) apoptosis.

protein immunoassay was performed in RPE cells injected with spermidine. Results showed that ALDH could significantly inhibit oxidative stress-induced RPE degeneration [114].

5.5.2. GSH and AMD. Cigarette smoking is the most important environmental risk factor for the occurrence of AMD, and damage to the RPE may be the cause of AMD. Exposure of RPE cells to cigarette smoke extract (CSE) or hydroquinone (HQ) leads to oxidative damage and apoptosis, characterized by cell size reduction and nuclear condensation. Evidence of oxidative damage also includes increased LPO (4-HNE) and mitochondrial superoxide production, and decreased intracellular GSH. Moreover, exogenous administration of the antioxidant GSH prevents oxidative damage of the RPE induced by cerebrospinal fluid [115].

5.6. Other Diseases linked to 4-HNE. The aldehyde compound 4-HNE forms adducts with free amino and mercaptan groups of proteins in the blood vessels causing the accumulation of 4-HNE adducts, which gradually leads to cell dysfunction, tissue damage, and atherosclerosis-related diseases. By forming the 4-HNE-apoB (apolipoprotein B) adducts, 4-HNE deviates from the low-density lipoprotein (LDL) metabolism involving scavenger receptor pathway of macrophages to the formation of foam cells, thus promoting atherogenesis. The 4-HNE adducts accumulate in the lipid necrotic core of advanced atherosclerotic lesions and may locally participate in macrophage and smooth muscle cell apoptosis, resulting in plaque instability and rupture, thus increasing the risk of atherosclerotic thrombosis events [116].

Immunohistochemistry and confocal immunofluorescence studies showed that 4-HNE-His adducts accumulated in the intima, media, and adventitia of the human aorta in an age-related manner and were mainly expressed in the smooth muscle cells. Therefore, the secondary product of LPO, especially 4-HNE, plays a complex role in elastin

Table 1: Pharmacological measures of 4-HNE detoxification.

Disease	Protein	Detoxification	Reference
AD	apoE (Cys)	apoE (Cys)-4-HNE prevents neuronal protein damage	[70–72]
	Alda-1	Activate ALDH2 and ALDH2∗2	[74]
	GSH	Eliminate the toxic aldehyde	[75]
PD	ALDHA1	Reduce the content of 4-HNE-protein and inhibiting cytotoxicity	[89, 90]
	PKM2	Regulate the synthesis of GSH	[91]
Cancer	ALDH2	High levels of 4-HNE increase GSH and NRF2	[100]
		Consume 4-HNE	[102]
	Hcy	Induce GSH mediate Nrf2 protect HepG2	[104]
ARHL	GSTA1, GSTA2	Prevent 4-HNE formation	[9]
	GSTA4, GSTA5	GSH-4-HNE	[9]
AMD	ALDH	Inhibit retinal epithelial degeneration	[114]
	GSH	Prevent oxidative damage of RPE	[115]

homeostasis, vascular wall remodeling, and atherosclerosis during aging [117].

Rossin et al. [118] found that 4-HNE and oxysterols exert their action intracellularly, by altering the redox balance of normal cells and activating antioxidant response signals. 4-HNE induces the cell signaling pathways of proliferation and survival which drive cells towards tumor resistance, developing colorectal cancer (CRC) and Inflammatory Bowel Disease (IBD).

6. Conclusions

Toxic aldehydes such as 4-HNE, produced via LPO due to the accumulation of ROS, play an important role in the development of aging-related diseases (Figure 3 and Table 1). 4-HNE can covalently bind to the membranes of key neurons that leads to the formation and aggregation of neurofibrillary tangles, and ultimately results in neuronal protein dysfunction, death, cognitive impairment, language disorders, and other neurodegenerative symptoms that collectively aggravate the risk of AD and PD. The electrophilicity of C3 in 4-HNE causes 4-HNE to constitutively bind to proteins and DNA, and this tendency leads to the compromised genome and protein function that gives rise to tumorigenesis and pathogenesis of various aging-related diseases. Evidently, the accumulation of 4-HNE-protein adducts *in vivo* affects the regulation of Nrf2/Keap1/ARE signaling pathways. This review also found that the toxic effect of 4-HNE on lipoprotein is related to the formation of atherosclerosis, and its response to collagen may be the cause of cardiovascular tissue sclerosis. 4-HNE can activate various molecules, such as NF-κB and NOX4, to induce RPE apoptosis, lysosomal imbalance, and lipofuscin production, resulting in photoreceptor cell destruction and consequently, age-related visual impairment such as AMD. Studies have found that ALDH and GSH pathways have a potential detoxification effect on toxic aldehydes by delaying the development of aging-related diseases caused by 4-HNE. Therefore, 4-HNE may be a potential target for clinical therapy aiming at delaying aging-related diseases.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest.

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Research Article

Inflamm-Aging-Related Cytokines of IL-17 and IFN-γ Accelerate Osteoclastogenesis and Periodontal Destruction

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Periodontal disease (PD), as an age-related disease, prevalent in middle-aged and elderly population, is characterized as inflammatory periodontal tissue loss, including gingival inflammation and alveolar bone resorption. However, the definite mechanism of aging-related inflammation in PD pathology needs further investigation. Our study is aimed at exploring the effect of inflamm-aging-related cytokines of interleukin-17 (IL-17) and interferon-γ (IFN-γ) on osteoclastogenesis *in vitro* and periodontal destruction in *vivo*. For receptor activator of nuclear factor-κB ligand- (RANKL-) primed bone marrow macrophages (BMMs), IL-17 and IFN-γ enhanced osteoclastogenesis, with the expression of osteoclastogenic mRNA (TRAP, c-Fos, MMP-9, Ctsk, and NFATc1) and protein (c-Fos and MMP-9) upregulated. Ligament-induced rat models were established to investigate the role of IL-17 and IFN-γ on experimental periodontitis. Both IL-17 and IFN-γ could enhance the local inflammation in gingival tissues. Although there might be an antagonistic interaction between IL-17 and IFN-γ, IL-17 and IFN-γ could facilitate alveolar bone loss and osteoclast differentiation.

1. Introduction

Periodontal disease (PD) is a chronic inflammatory disease that occurs in the periodontal support tissue caused by periodontopathic bacteria. It is characterized by gingival inflammation and destruction of alveolar bone [1, 2]. The health of the periodontal tissues is not only directly linked to oral diseases but also implicated in system-wide health, but the pathogenesis of periodontitis is still unidentified. Recent studies have reported that PD is highly prevalent in adults and the disease severity increases with age. More than 64% of adults aged ≥65 suffered from moderate/severe periodontitis, causing a huge economic burden worldwide [3, 4].

Inflamm-aging refers to a proinflammatory state which is chronic, systematic, and controllable in the aging process [5]. The proinflammatory state accelerates the aging process, and in turn, the aging of the body aggravates the development of inflammatory response, thus forming a vicious circle [6]. Inflamm-aging is closely associated with many chronic diseases, such as periodontitis, type 2 diabetes, insulin resistance, atherosclerosis, osteoporosis, and arthritis [7–9]. During aging, chronic periodontal inflammation is often accelerated with the aging of tissues and organs [10].

Recent studies on the mechanisms of inflamm-aging pay attention to oxidative stress, DNA damage, autophagy, and inflammatory cytokines [11–13]. In the cellular immune

response, Th1/Th2/Th17/Treg responses and cytokines play important roles in inflamm-aging induced by chronic inflammation [6, 14–17]. During initiation and progression of periodontitis, Th1 cell and mainly cytokine interferon-γ (IFN-γ) were previously regarded as having key importance in bone loss related to inflammation [18, 19]. However, accumulating pieces of evidences have shown that the inflammation of PD may be dominantly mediated through Th17 cells and its main factor interleukin-17A (IL-17) [20, 21]. We previously found significantly higher levels of IL-17 and IFN-γ in plasma, gingival crevicular fluid, and gingival biopsy samples of patients with chronic periodontitis than healthy individuals [22]. However, how IL-17 and IFN-γ, as inflamm-aging-related cytokines, regulate the initiation and development of PD remains unclear.

IL-17 has been demonstrated to have multiple effects on the process of bone destruction related to inflammation [23–25]. IL-17 can act on macrophages and synergistically increase the production of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 [26]. IL-17 can also recruit neutrophils to the inflammatory sites and enhance granulopoiesis to activate neutrophil-mediated inflammation [27]. Our former *in vivo* study showed that there was an increased number of osteoclasts and alveolar bone resorption in IL-17-treated periodontitis rat model [28]. Hence, IL-17 is a potent mediator to bridge aging-related inflammation with alveolar bone.

IFN-γ also plays an important part in inflammation and osteoclastogenesis. Since it has a complex role in physiological and pathological conditions, it is difficult to define IFN-γ as a proinflammatory or anti-inflammatory cytokine [27]. IFN-γ mutant mice challenged with *Porphyromonas gingivalis* showed less alveolar bone loss compared with the sham-operated mice [29], indicating its proresorptive effect on alveolar bone. IFN-γ has a dual effect in osteoclasts depending on the level of IFN-γ, receptor activator of nuclear factor-κB ligand (RANKL), and the stage of osteoclast differentiation [30]. Several studies reported that IFN-γ could exert an inhibitory effect in the early stage of osteoclast differentiation and a stimulatory effect in the late stage of osteoclast maturation [27, 30, 31].

However, the role of IFN-γ combined with IL-17 on the process of PD *in vivo* is yet to be elucidated. At present, there are relatively few reports on the mechanism of inflamm-aging in periodontitis, and how to mitigate periodontitis through regulating inflamm-aging is yet pending further study. Hence, the present study is aimed at investigating the effect of inflamm-aging-related cytokines of IL-17 or/and IFN-γ on osteoclast differentiation *in vivo*, as well as gingival inflammation and alveolar bone loss in the ligature-induced periodontitis rat model.

2. Materials and Methods

2.1. Cell Culture and Osteoclast Induction. Bone marrow macrophages (BMMs) were collected as described previously [28]. Briefly, C57BL/6 mice (6-8 weeks old) were anesthetized with sodium pentobarbital (150 mg/kg, intraperitoneally) and then sacrificed through cervical dislocation. The tibiae and femora were dissected with the ends being removed. Then, a syringe was used to flush the marrow cavity with α-MEM medium (Gibco, USA) supplemented with 10% FBS (fetal bovine serum, Gibco, USA), 100 U/μl penicillin (TBD Science, China), 100 U/μl streptomycin (TBD Science, China), and 30 ng/ml macrophage colony-stimulating factor (M-CSF; R&D, Minneapolis, MN, USA). The mixed cells were incubated at the environment of 37°C and 5% CO_2 for 3 days. The adherent cells were considered as BMMs and used for the following *in vitro* studies.

To induce osteoclasts, BMMs were seeded in 48-well plates at the density of 3×10^4 cells/well in the presence of M-CSF (30 ng/ml) and RANKL (20 ng/ml, R&D, Minneapolis, MN, USA). Different concentrations of IL-17 (0.1, 1, and 10 ng/ml, PeproTech, Rocky Hill, NJ, USA) and IFN-γ (0.02, 0.2, and 2 ng/ml, PeproTech, Rocky Hill, NJ, USA) were added on the third day or the sixth day after seeding. The culture media were changed every other day.

2.2. Ligature-Induced Periodontitis Rat Model. A total of 75 eight-week-old Sprague-Dawley rats (200 ± 20 g) were obtained from the China Experimental Animal Center (Hangzhou, China). The rats were housed in individual cages under the temperature of 21 ± 2°C with 12 h light-dark cycles and humidity of 50 ± 5%. They were fed with high-glucose laboratory food and high-glucose tap water *ad libitum*. The protocol of this rat experiment was approved by the Animal Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine (No. 2017-052).

Subjects were divided randomly into 5 groups: NC (normal control) group, NS (normal saline) group, IL-17 group, IFN-γ group, and IL-17+IFN-γ group. Each group included five rats. To establish an experimental periodontitis rat model, as previously described [28], after the anesthetization procedure with sodium pentobarbital (40 mg/kg) via intraperitoneal injection, 2 mm orthodontic ligatures (West Lake Barr, Hangzhou West Lake Biomaterials Co., China) were fixed around the cervical margins around the bilateral maxillary first molars. In the NC group, sham treatment was applied. Afterward, the rats received injection of 20 μl normal saline, 20 μl IL-17 (5 μg/ml), or/and IFN-γ (1 μg/ml) into the soft tissue around the maxillary bilateral first molars under anesthesia every other day, respectively. The rats in the NC group did not receive any injection. After 1, 2, and 4 weeks, rats were humanely sacrificed under anesthesia.

2.3. Histological Observation. The right sides of the maxillary first molars and the surrounding tissues of the 1-week and 2-week samples were harvested and fixed in 4% phosphate-buffered paraformaldehyde (Boster Biological Technology, Wuhan, China) (pH = 7.2) for 48 h and then decalcified in 10% EDTA solution for 8 weeks. The decalcified samples were dehydrated in ethanol and embedded in paraffin. Subsequently, the mesiodistal slices with thickness of 5 μm were obtained and stained with hematoxylin and eosin (HE). The number of inflammatory cells, mainly neutrophils, and lymphocytes in the interproximal regions between the first and second molars was counted according to their

morphologies. The mean number of five randomly selected fields at 400x magnification was used for each section.

2.4. Immunohistochemistry and Tartrate-Resistant Acid Phosphatase (TRAP) Staining. Dewaxed and rehydrated sections were treated with 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. After washing in PBS, sections were incubated with primary polyclonal antibodies against IL-6 (1 : 100; Proteintech, Chicago, IL, USA), IL-1β (1 : 100; Proteintech, Chicago, IL, USA), or TNF-α (1 : 100; Proteintech, Chicago, IL, USA) overnight at 4°C. The sections were then washed and treated with secondary antibodies (Boster Biological Technology, Wuhan, China). Afterward, these sections were stained using a 3,3′-diaminobenzidine kit (Boster Biological Technology, Wuhan, China) and then counterstained with hematoxylin.

Osteoclasts were classified by the specific marker tartrate-resistant acid phosphatase (TRAP) using a kit from Sigma (St. Louis, MO, USA). Active osteoclasts were identified when TRAP-positive cells contained three or more nuclei.

IL-6-positive, IL-1β-positive, and TNF-α-positive cells and active osteoclasts in the interradicular regions of the first molars were counted at 400x magnification. The mean number of the five randomly selected fields was recorded for each section, and three sections of each rat were counted.

2.5. Micro-Computed Tomography (Micro-CT) Scanning. The 4-week samples of molars were scanned using a micro-CT scanning machine (Scanco Medical AG, Bassersdorf, Switzerland). The micro-CT parameters were set as follows: pixel size, 10 × 10 μm; slice thickness, 10 μm; voltage, 70 kV; and electrical current, 200 μA.

The images were reconstructed to generate three-dimensional (3D) models. Bone loss was measured as previously described [32], which was the distance from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) along the buccal and lingual long axis of the maxillary first molars. Six sites were detected per tooth for each rat.

A circle with fixed diameter was selected as the observation area between the mesial and distal root of the first molar on the transverse section. The interradicular regions of the maxillary first molars were selected for bone volume per tissue volume (BV/TV) analysis.

2.6. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis. RT-qPCR analysis was applied to evaluate the gene expression of osteogenic markers in BMMs and gingival samples. Cells were harvested using TRIzol reagent (Invitrogen; Thermo, Waltham, MA, USA) two days after adding IL-17 or/and IFN-γ. Gingival tissue with a width of 2 mm below the gingival margin of the left first molars of 2-week and 4-week samples was excised and grinded. Total cellular RNA was extracted following treatment with TRIzol reagent on ice. A total of 1 μg RNA was reverse-transcribed to generate single-stranded cDNA using the PrimeScript Reverse Transcription Master Mix Kit (Takara, Otsu, Japan). The reverse transcription protocol was as follows: 37°C for 15 min, 85°C for 5 sec and terminating at 4°C. The expression level of target genes was quantified using a SYBR Premix Ex Taq™ II kit (Takara, Otsu, Japan) on the StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo, Waltham, MA, USA). The primer sequences are listed in Table S1.

The amplification procedure was performed for 40 cycles at 95°C (30 seconds), 95°C (5 seconds), and 60°C (30 seconds). The relative expression level of target genes was calculated by the relative quantitative method ($2^{-\Delta\Delta Cq}$) and normalized to the mouse GAPDH gene.

2.7. Western Blotting Analysis. Western blotting analysis was applied to evaluate the protein expression in BMMs which were harvested by cell lysate buffer (Thermo Fisher Scientific, USA) two days after adding IL-17 or/and IFN-γ. After being quantified by bicinchoninic acid kit (Thermo Fisher Scientific, USA), equal amounts of total protein (20 μg) were resolved by SDS-PAGE on a 10% gel and transblotted onto the polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were then blocked with 5% skim milk (BD Difco, USA) for 1 h at room temperature. Anti-GAPDH (1 : 1000, Fdbio Science, China), c-Fos (1 : 1000, Proteintech, Chicago, IL, USA), and MMP-9 (1 : 1000, Abcam) were incubated overnight at 4°C. Membranes were washed and incubated with alpaca anti-rabbit antibody (1 : 50000, HuaBio, China) and peroxidase-conjugated goat anti-mouse antibody (1 : 5000, Fudebio, China) at room temperature for 1 h. After washing, immunoreactive bands were visualized using an ECL kit (Fudebio, China) using chemiluminescence imaging apparatus (Tanon 5200 Multi, Tanon, China).

2.8. Statistical Analysis. Each experiment was performed in triplicate. All quantitative data are presented as mean ± standard deviation. Statistical analyses between groups were performed by one-way analysis of variance (ANOVA), followed by Tukey-t-test using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Statistically significant difference was considered when $p < 0.05$.

3. Results

3.1. IL-17 and IFN-γ Enhance Osteoclast Differentiation of Mouse Bone Marrow Macrophages. To evaluate the effects of IL-17 or/and IFN-γ on osteoclast differentiation, BMMs were cultured with different concentrations of IL-17 (0.1, 1, and 10 ng/ml) or/and IFN-γ (0.02, 0.2, and 2 ng/ml). At the setting of condition 1, IL-17 or/and IFN-γ were added on day 6 when some of the BMMs have been preliminarily induced into minor multinucleated cells. The formation of TRAP-positive multinucleated cells is the hallmark of osteoclast formation. The number of osteoclasts was counted, and the area of osteoclasts was calculated (Figures 1(l) and 1(m) and Figure S1). The result showed significantly larger TRAP-positive cells when adding IL-17 or IFN-γ compared to the control group. The groups of median concentration of IL-17 (1 ng/ml) and IFN-γ (0.2 ng/ml) showed the best differentiation capacity of BMMs. However, when adding both IL-17 and IFN-γ, the formation of giant mature

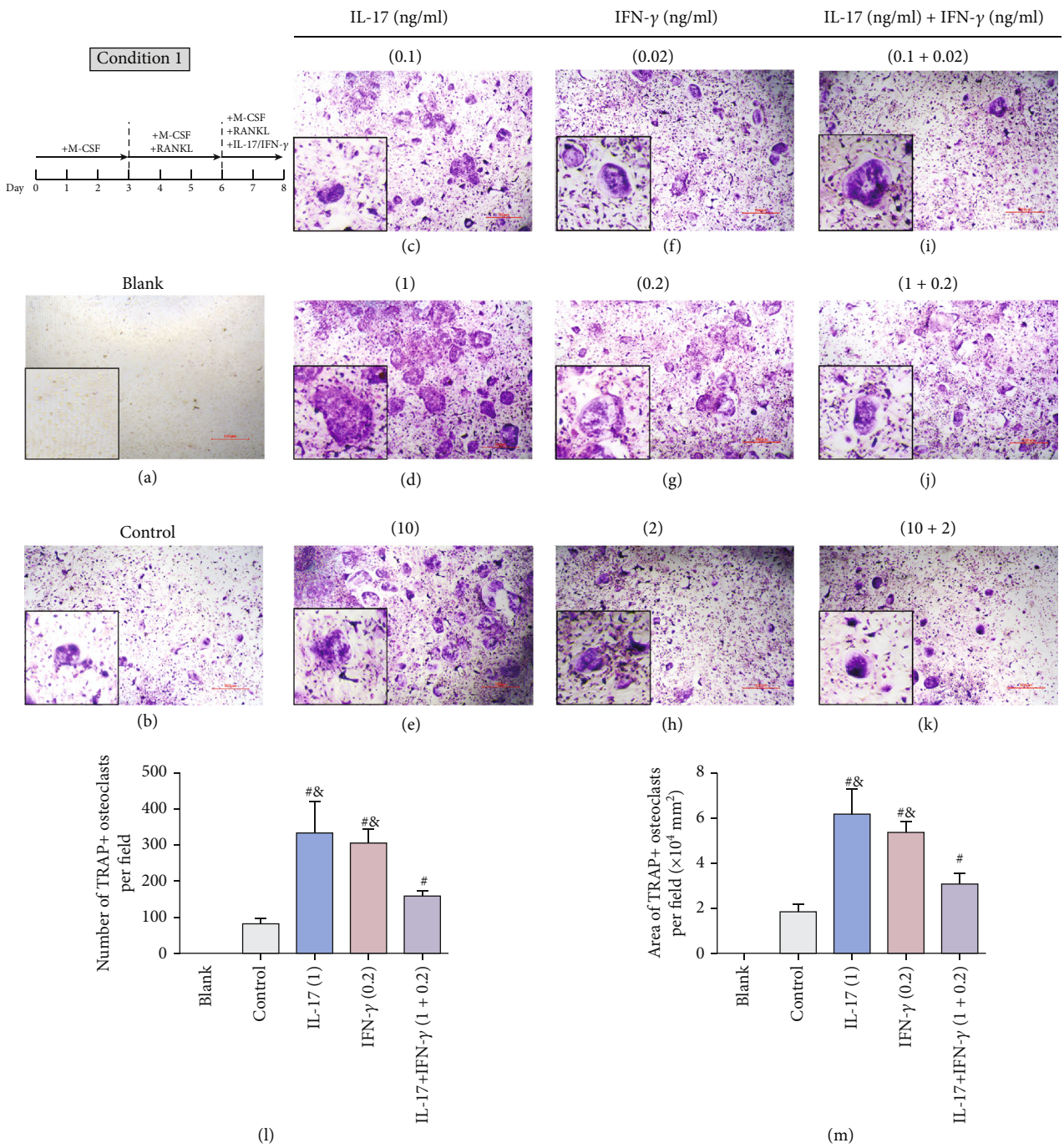

Figure 1: Effect of IL-17 or/and IFN-γ on differentiation of preosteoclasts. (a–k) In condition 1, BMMs were cultured with M-CSF (30 ng/ml) for 3 days and then with 20 ng/ml RANKL for another 3 days. On day 6, IL-17 or/and IFN-γ of different concentrations was added. (l, m) The results of TRAP staining of each group are presented (scale bar = 500 μm). Osteoclasts were recognized as TRAP-positive multinucleated cells (≥3 nuclei). (l) The number of TRAP-positive osteoclasts and (m) the area of TRAP-positive osteoclasts were counted and presented (mean ± standard deviation, $n = 3$). $^{\#}p < 0.05$ compared with the control group; $^{\&}p < 0.05$ compared with the IL-17+IFN-γ group.

osteoclasts was significantly inhibited (Figures 1(i)–1(k)) compared with the respective groups of IL-17 or IFN-γ alone.

Interestingly, when IL-17 or IFN-γ was added together with RANKL at the beginning (condition 2), the number and area of TRAP-positive cells were not significantly upregulated compared to the control group (Figure S2), indicating that the early exposure to IL-17 or/and IFN-γ might attenuate the effect of RANKL on the differentiation of osteoclasts.

3.2. IL-17 and IFN-γ Upregulated the Expression of Osteoclastogenic mRNA and Protein In Vitro. In an attempt to explore the explanation for the stimulatory effect of IL-17 and IFN-γ on osteoclastogenesis of BMMs, the

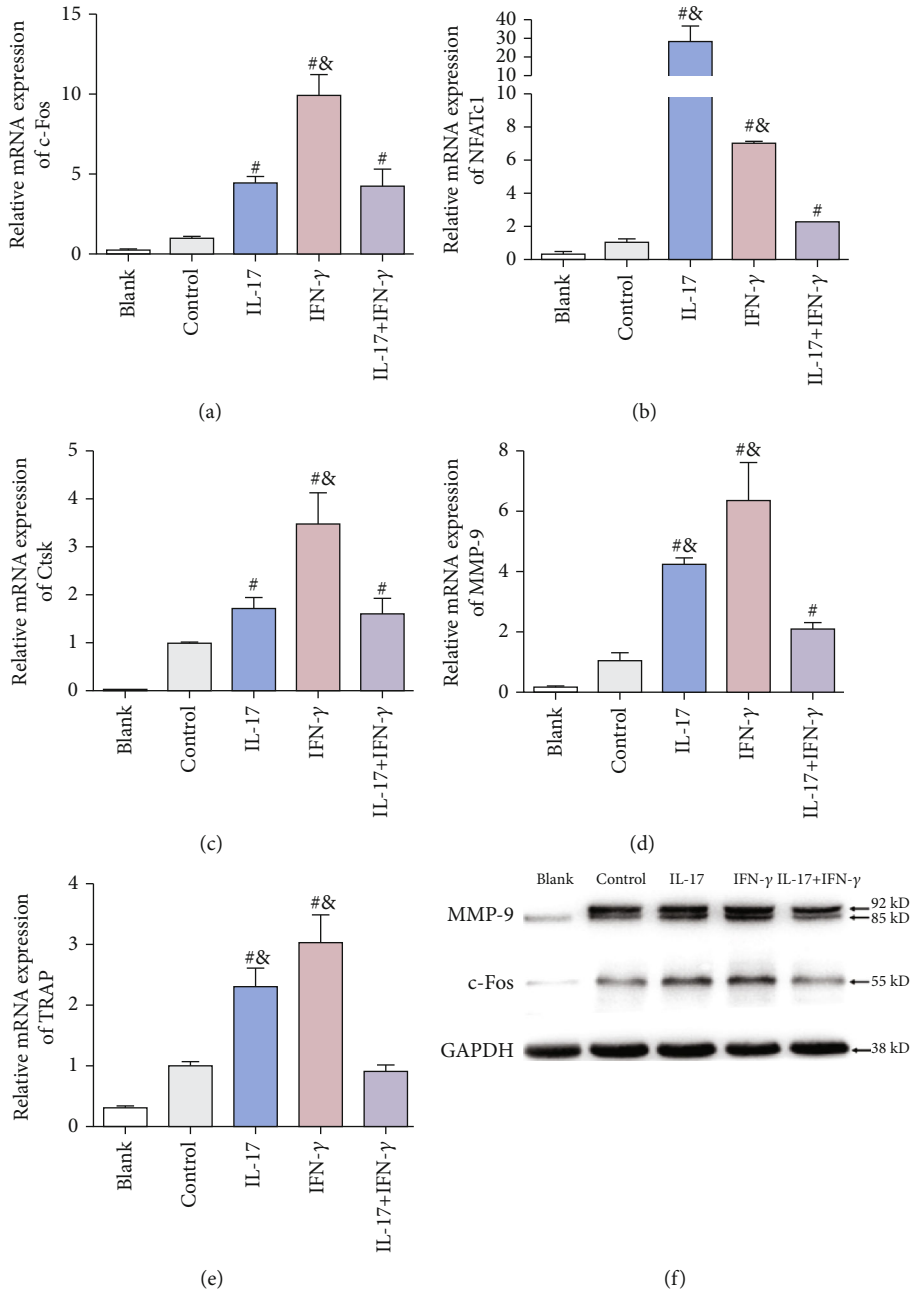

Figure 2: Effects of IL-17 (1 ng/ml) or/and IFN-γ (0.2 ng/ml) on the expression of osteoclastogenic genes *in vitro*. The mRNA expression of osteoclast-related genes (a) c-Fos, (b) NFATc1, (c) Ctsk, (d) MMP-9, and (e) TRAP was detected with different concentrations of IL-17 or/and IFN-γ using RT-qPCR. Data were standardized to GAPDH expression and shown as a fold change relative to the control group (mean ± standard deviation, $n = 3$). $^{\#}p < 0.05$ compared with the control group; $^{\&}p < 0.05$ compared with the IL-17+IFN-γ group.

osteoclastogenic gene expression was analyzed using RT-qPCR, including c-Fos, nuclear factor of activated T cells 1 (NFATc1), cathepsin K (Ctsk), matrix metallopeptidase 9 (MMP-9), and TRAP. The mRNA expression of c-Fos, NFATc1, Ctsk, MMP-9, and TRAP was significantly upregulated in the presence of IL-17 or IFN-γ compared to the control group (Figure 2, Figure S3). Consistent with the result of TRAP staining, the group of IL-17 (1 ng/ml) and IFN-γ (0.2 ng/ml) showed the strongest elevation of these osteoclastogenic genes. However, the combined addition of IL-17 and IFN-γ displayed significantly lower mRNA level of osteoclastogenic genes, suggesting the antagonistic effect between IL-17 and IFN-γ.

As for the results of Western blotting analysis, the protein level of c-Fos and MMP-9 was significantly increased in the presence of IL-17 (1 ng/ml) or IFN-γ (0.2 ng/ml) compared to the control group (Figure 2). Similarly, the combined use of IL-17 and IFN-γ showed decreased proosteoclastogenic effect.

3.3. IL-17 and IFN-γ Induced Local Gingival Inflammation in Experimental Periodontitis Rat Model. To investigate the

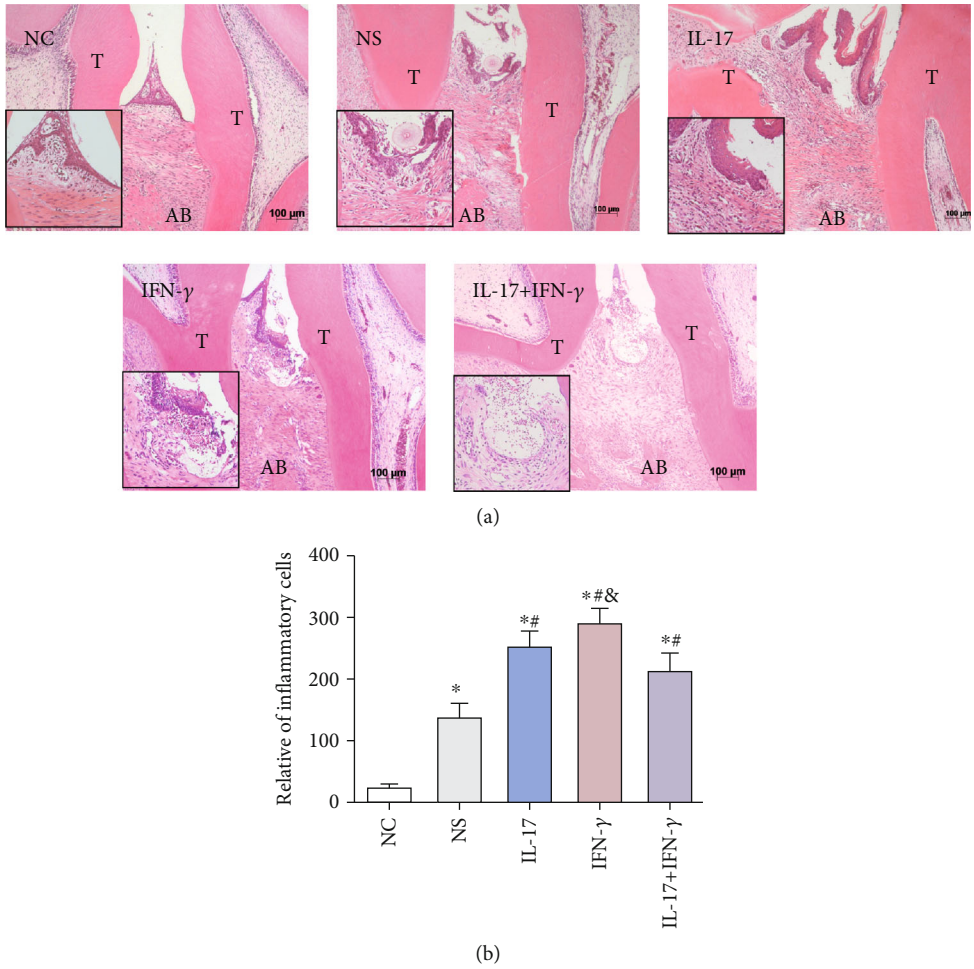

Figure 3: Role of IL-17 (5 μg/ml) or/and IFN-γ (1 μg/ml) on local gingival inflammation in an experimental periodontitis rat model. (a) HE staining showed the periodontal morphology in the interradicular regions of the first maxillary molars of the NC, NS, IL-17, IFN-γ, and IL-17 +IFN-γ groups (scale bar = 100 μm). (b) Quantitative analysis was performed on the number of inflammatory cells of each group (mean ± standard deviation, $n = 5$). $^{*}p < 0.05$ compared with the NC group, $^{\#}p < 0.05$ compared with the NS group, and $^{\&}p < 0.05$ compared with the IL-17+IFN-γ group. T: tooth; AB: alveolar bone.

effect of IL-17 and/or IFN-γ on the periodontal tissue *in vivo*, we established a rat model of ligature-induced periodontitis and the respective cytokines were locally injected every two days. After 2 weeks, HE staining showed normal gingiva structure with well-aligned periodontal ligament fiber orientation and few inflammatory cells in the NC group (Figure 3). However, in the ligature-induced groups (NS group, IL-17 group, IFN-γ group, and IL-17+IFN-γ group), the periodontal ligament fibers were disorderly arranged and the alveolar bone lost by varying degrees. Histology results confirmed the typical periodontitis symptoms in the experimental periodontitis group. Many inflammatory cells mainly polymorphonuclear leukocytes invaded the connective tissue as well as the epithelium layer in the NS group, the IL-17 group, the IFN-γ group, and the IL-17+IFN-γ group compared to the NC group (Figure 3). After calculating the inflammatory cells, the IL-17group and the IFN-γ group presented significantly more inflammatory cells than the NS group ($p < 0.05$). Nevertheless, the combined injection of IL-17 and IFN-γ resulted in less inflammation than groups that were injected IL-17 or IFN-γ solely ($p < 0.05$).

3.4. IL-17 and IFN-γ Enhance the Proinflammatory Cytokine Expression in Interradicular Regions of the First Maxillary Molars and in Gingival Tissues In Vivo. As the immunohistochemistry results show (Figures 4(a)–4(c)), the immunoreactive cells of IL-1β, TNF-α, and IL-6 were characterized by brown-colored cytoplasm in immunohistochemical staining. The cytokines IL-6 and IL-1β were found having higher expression in fibroblasts and inflammatory cells in the ligature-induced gingival zone, while TNF-α was found having higher expression in the inflammatory cells in the bone resorption zone. Compared with the NC group, IL-1β, TNF-α, and IL-6 were observed significantly increased in the NS, IL-17, IFN-γ, and IL-17+IFN-γ groups ($p < 0.05$). IL-1β and TNF-α were found having significantly higher expression in the IL-17 and IFN-γ groups than in the NS group ($p < 0.05$), but with significantly lower expression in the IL-17+IFN-γ group than in the IL-17 or IFN-γ group ($p < 0.05$). However, the IL-6 results were comparable among the ligature-induced groups ($p > 0.05$).

The mRNA expression of proinflammatory cytokines (IL-1β, TNF-α, and IL-6) was examined by RT-qPCR

(Figures 4(g)–4(i)). After the periodontitis model establishment for 1 week, significantly increased mRNA expression of IL-1β, TNF-α, and IL-6 was observed in the IL-17 and IFN-γ groups than in the NS group ($p < 0.05$). However, after combined injection of IL-17 and IFN-γ, these proinflammatory cytokines showed lower expression than the IL-17 group or the IFN-γ group ($p < 0.05$). After 2 weeks, the same tendency of IL-1β and TNF-α expression among groups was observed in gingival samples. On the contrary, IL-6 level was decreased, and the IL-17 and IFN-γ groups showed even lower IL-6 expression than the NS group ($p < 0.05$).

3.5. IL-17 and IFN-γ Facilitate Alveolar Bone Loss and Osteoclast Differentiation in Experimental Periodontitis Rat Model. We used micro-CT to further detect the alveolar bone level around teeth after 4-week induction, and the reconstructed three-dimensional images of these groups are presented in Figure 5(a). The CEJ-ABC results of the first maxillary molar were 0.49 ± 0.1 mm, 0.74 ± 0.12 mm, 1.09 ± 0.16 mm, 1.42 ± 0.27 mm, and 1.17 ± 0.13 mm for the NC, NS, IL-17, IFN-γ, and IL-17+IFN-γ groups, respectively. The cytokine injection groups showed more alveolar bone loss around the first maxillary molar than the NS group. In addition, the combined injection of IL-17 and IFN-γ resulted in significantly less bone loss than the IFN-γ group ($p < 0.05$) but was comparable with the IL-17 group ($p > 0.05$).

When comparing the bone structure parameters, the ligature-induced groups exhibited decreased bone volume per tissue volume (BV/TV), and the injection of IL-17 and IFN-γ could accelerate this tendency.

The formation of TRAP-positive multinucleated cells is regarded as the hallmark of osteoclast differentiation. TRAP staining exposed more TRAP-positive cells in the ligature-induced groups than the NC group, as reflected by the increased quantity of multinucleated giant cells. After calculating their number in the interdental zone between the first molar and the second molar, the results showed more TRAP-positive multinucleated cell formation in the IL-17 group (27.67 ± 2.08), the IFN-γ group (30.00 ± 2.65), and the IL-17+IFN-γ group (22.33 ± 2.52) than in the NC group (4.67 ± 1.53) ($p < 0.05$) (Figures 5(d) and 5(e)).

4. Discussion

Inflammation is a complex response to pathogen infection and tissue injury in host immunity. Various mechanisms such as autophagy and reactive oxygen species (ROS) mediate the production of proinflammatory factors, and the excessive proinflammatory response leads to inflammatory aging [11, 12]. Periodontal pathogen-induced host immune response and the overexpression of cytokines exert a profound impact on aggravating gingival inflammation and activating bone destruction [33]. Despite increasing awareness of periodontitis impact on general health, limited progress has been made in understanding the immunological mechanisms to control aging-related inflammation. Our data demonstrated that the use of IL-17 or IFN-γ could enhance osteoclastogenesis *in vitro* and exaggerate gingival inflammation and alveolar bone loss *in vivo*. However, there might be an antagonistic interaction between IL-17 and IFN-γ.

In the inflamm-aging process, given the vital role of the innate immune system in maintaining periodontal tissue homeostasis through regulated production of proinflammatory cytokines, excessive inflammation can impact bone metabolism. Our *in vitro* study showed that IL-17 and IFN-γ could increase the number and the area of TRAP-positive multinucleated cells as well as the expression of osteoclastogenic markers (c-Fos, NFATc1, Ctsk, MMP-9, and TRAP), indicating their proosteoclastogenic capability. However, early exposure to RANKL could render BMMs resistant to IL-17 and IFN-γ and result in less formation of mature osteoclasts, indicating the timing of the occurrence of RANKL played an essential role. Both IL-17 and IFN-γ played a stimulatory role on the RANKL-induced preosteoclasts rather than uninduced BMMs. Our previous research showed that IL-17 could enhance osteoclast differentiation via activation of autophagy [28]. In this study, we found that simultaneous treatment of RANKL and IL-17 could reverse the proosteoclastogenic capability of IL-17. It is reported that IL-17A could suppress the expression of osteoclastogenic proteinases and osteoclast differentiation, which is attributed to the high concentrations of IL-17A [34]. Huang et al.'s finding also showed that IFN-γ had a dual effect on osteoclastogenesis in mouse macrophage cell line RAW264.7, depending on the stage of osteoclast precursors when IFN-γ was added [31]. In the early stage when macrophages have not encountered sufficient RANKL, IFN-γ potently induces macrophage activation; with pretreatment of RANKL that induces macrophages into preosteoclasts, IFN-γ could enhance the maturation of preosteoclasts [31]. Of note, the effect of IL-17 and IFN-γ on osteoclastogenesis depends on their concentration due to complex signal pathway regulation. In our study, IL-17 (1 ng/ml) and IFN-γ (0.2 ng/ml) showed the most significant effect for osteoclastogenesis, but the trend is not dose dependent. Some researchers reported similar results. Adamopoulos et al. [35] found that exposure to IL-17A could promote peripheral blood mononuclear cells (hPBMCs) to differentiate into functional osteoclasts, while this effect is not dose dependent. Among the concentration setting of 0.1, 1, 10, and 100 ng/ml, 1 ng/ml of IL-17A showed the most significant induction [35]. Another study by Ke et al. [36] found that a low concentration of IL-17A (0.5 ng/ml) could facilitate autophagy of osteoclast precursors via the RANKL-JNK signaling pathway, thus enhancing RANKL-induced osteoclastogenesis. However, treatment with a high concentration of IL-17A (5-50 ng/ml) might inhibit autophagy and decrease osteoclast formation [36]. As for the role of IFN-γ on osteoclastogenesis, Huang et al. [31] showed that after pretreatment of RANKL (50 ng/ml), low concentration of IFN-γ (0.1, 1.0 ng/ml) could enhance osteoclast formation. However, higher concentration of IFN-γ (10, 100 ng/ml) markedly inhibited osteoclastogenesis [31]. Moreover, Kim et al. [37] found that IFN-γ (0, 0.1, 0.5, and 1.0 ng/ml) could enhance osteoclast fusion at a dose-dependent manner when BMMs were treated with M-CSF (20 ng/ml) and RANKL (50 ng/ml) for 3 days before IFN-γ addition. If IFN-γ (1 ng/ml) was added together with M-CSF and RANKL, it

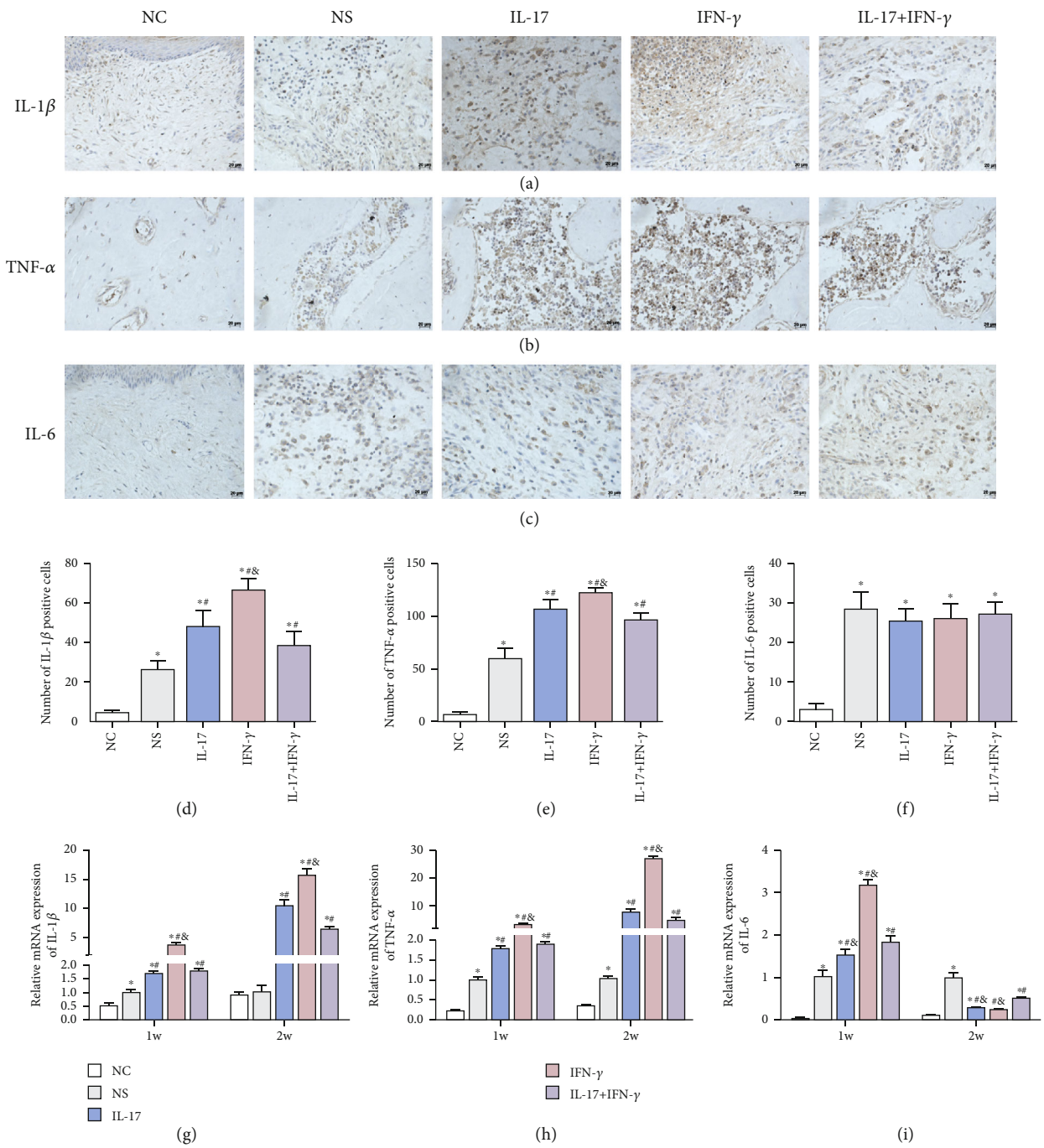

Figure 4: Effect of IL-17 (5 μg/ml) or/and IFN-γ (1 μg/ml) on proinflammatory cytokine expression *in vivo*. Immunohistochemical staining was used to examine the level of (a) IL-1β, (b) TNF-α, and (c) IL-6 expression in gingival tissues (scale bar = 20 μm). Quantitative analysis results of (d) IL-1β-, (e) TNF-α-, and (f) IL-6-positive cells are shown. The mRNA level of (g) IL-1β, (h) TNF-α, and (i) IL-6 in gingival tissues after 1 week and 2 weeks was detected by RT-qPCR. Data were presented by mean ± standard deviation, $n = 5$. $^{*}p < 0.05$ compared with the NC group, $^{\#}p < 0.05$ compared with the NS group, and $^{\&}p < 0.05$ compared with the IL-17+IFN-γ group.

could exert an inhibitory effect, indicating that IFN-γ could induce the differentiation of RANKL-induced BMMs into multinucleated osteoclasts [37].

The effect of IL-17 and IFN-γ on periodontal gingival tissues and alveolar bone suggests their proresorption roles for periodontitis *in vivo*, which might partly be explained by their stimulatory effect on IL-1β, TNF-α, and IL-6. Salvioli et al. found that the expression levels of TNF-α, IL-1, and IL-6 and other cytokines increased with aging [38]. These cytokines, also known as senescence-associated secretory phenotype (SASP), are important pathogenic factors of inflamm-aging. Emerging experimental and clinical studies have demonstrated that TNF-α, IL-1β, and IL-6 are associated with the initiation and progression of periodontitis [39–42]. In this study, two weeks after the model establishment, the soft tissue lesions in the induced groups were

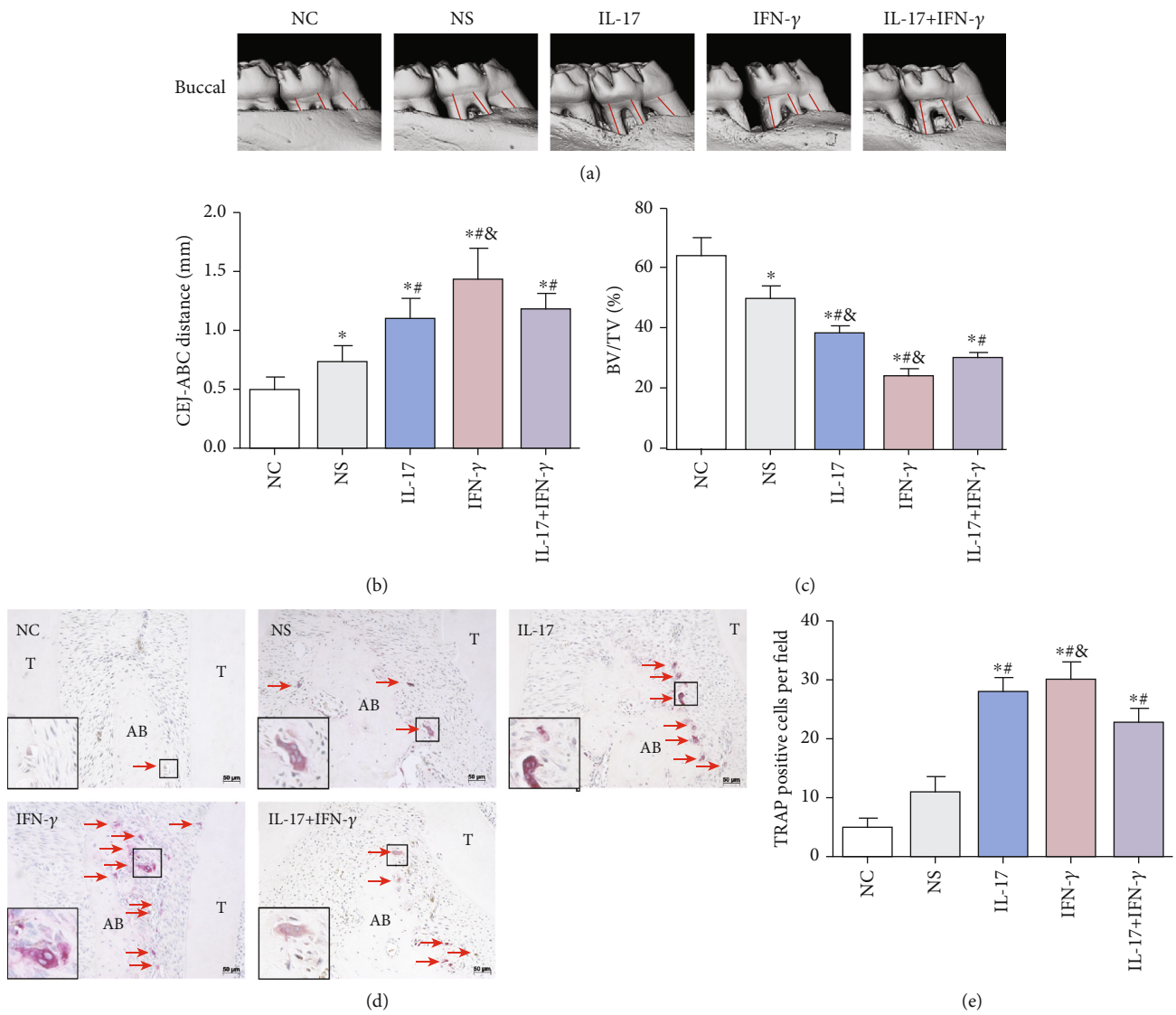

Figure 5: Effect of IL-17 (5 μg/ml) or/and IFN-γ (1 μg/ml) on alveolar bone loss and osteoclast differentiation. (a) Reconstructed micro-CT images showed the buccal view of the first maxillary molars. The distance from CEJ to ABC indicated bone loss, shown by the red lines. Quantitative analysis results of the (b) CEJ-ABC distance and (c) BV/TV are shown as mean ± standard deviation. (d) TRAP staining was used to evaluate the formation of osteoclasts (red arrows) for the NC, NS, IL-17, IFN-γ, and IL-17+IFN-γ groups (scale bar = 50 μm). (e) Quantitative analysis was performed on the number of TRAP-positive multinucleated cells in the interradicular regions of the first maxillary molars (mean ± standard deviation, $n = 5$). $^{*}p < 0.05$ compared with the NC group, $^{\#}p < 0.05$ compared with the NS group, and $^{\&}p < 0.05$ compared with the IL-17+IFN-γ group. ABC: alveolar bone crest; CEJ: cemento-enamel junction; BV: bone volume; TV: tissue volume; T: tooth; AB: alveolar bone.

characterized by hyperplasia and migration of gingival epithelium, inflammatory cell infiltration into gingival epithelium and periodontal ligament, and disruption of the periodontal ligament. Besides, local injection of IL-17 and IFN-γ could enhance the expression of mRNA and protein of TNF-α, IL-1β, and IL-6. Notably, the mRNA level of TNF-α and IL-1β of 2 weeks was higher than that of 1 week, while IL-6 mRNA expression showed decreased tendency. The possible reason might be that IL-6 played a proinflammatory and preosteoclastogenic role prior to TNF-α and IL-1β. This is in line with Pesic et al.'s findings that the level of TNF-α did not show significant changes during the early phase of fracture healing, while IL-6 increased statistically on the first day after intervention [43]. We speculated that the stimulating effects of IL-17 and IFN-γ on the expression of TNF-α and IL-1β might last longer than IL-6.

In parallel, both the height and density of alveolar bone were significantly decreased in the rat model after the administration of IL-17 or IFN-γ. Furthermore, the TRAP staining result of alveolar bone showed an increased number of multinucleated osteoclasts in the IL-17 and IFN-γ groups. Some *in vivo* studies found that the knockout of IL-17 or its receptor does not affect osteoclast numbers or bone mass in uninduced animal models but exerts a proosteoclastogenic role on parathyroid hormone- (PTH-) induced, ovariectomy- (OVX-) induced bone loss [44, 45]. These results indicated

that IL-17 might only affect bone under inflammatory conditions rather than normal physiological conditions. As for IFN-γ, the knockout of IFN-γ or its receptors in animal models could induce bone loss [46], while the effect of IFN-γ administration on bone mass depends on the dosage, frequency, and course [47]. These contradictory results might be attributed to the predominant effect on osteoclastogenesis, the balance between osteoblasts and osteoclasts, and the interaction between bone and immune system. Further researches are required to reveal the complicated roles of IL-17 and IFN-γ in different pathophysiological conditions.

Both in vivo and in vitro results indicated the antagonistic interaction between IL-17 and IFN-γ. As reported by Tu et al., both IL-17 and IFN-γ are required in combination for autoreactive T cells to cause severe damage in autoimmune gastritis [48]. IL-17 and IFN-γ also have the potential to induce proteoglycan-induced arthritis (PGIA), but IFN-γ suppresses IL-17 production, revealing that the effect of Th17 cells in PGIA requires alleviating IFN-γ-mediated IL-17 inhibition [49]. Therefore, we speculate that IL-17 production was weakened after IFN-γ interferes with the rat periodontitis model, and the proinflammatory role played by IL-17 decreased. Meanwhile, it should be noted that IL-17 in PGIA is pathogenic only when IFN-γ is ablated or reduced, indicating that IL-17 can play a proinflammatory role in PGIA if it is released from the inhibitory effect of IFN-γ [49]. This also suggested that IFN-γ might still inhibit the proinflammatory effect of IL-17 even though IL-17 and IFN-γ exist as proinflammatory factors during the progression of periodontitis.

We previously found that the expression of TRAF6, p-ERK, and p-p38 was all significantly upregulated in osteoclast precursor cells after IL-17 intervention, which suggested that the TRAF6/ERK/p38 signaling pathway might be involved in IL-17-mediated osteoclast differentiation [50]. In Li et al.'s study [51, 52] about the interaction between IL-17 and IFN-γ on the growth of mouse hepatoma Hepa1-6 cells (HCC), IL-17 or IFN-γ alone could significantly activate p38 MAPK and ERK1/2. It is noteworthy that the phosphorylation levels of p38 MAPK and ERK1/2 were significantly reduced after the combined intervention of IL-17 and IFN-γ, indicating that IFN-γ might antagonize the effect of IL-17 through the p38 MAPK/ERK1/2 signaling pathway. Considering our finding that there might be an antagonistic effect between IL-17 and IFN-γ, we speculated that this antagonistic effect might attribute to the regulation of p38MAPK and ERK signaling pathways during osteoclast differentiation, while the specific mechanism needs further verification.

5. Conclusion

In summary, the mechanism of inflamm-aging in the development of periodontitis is still in its nascent stages. Based on the key role of inflammatory cytokines in the process of inflamm-aging, our study revealed that IL-17 and IFN-γ could enhance osteoclastogenesis *in vitro*, while their proosteoclastogenic effect only played on the RANKL-primed BMMs. In ligament-induced experimental periodontitis rat model, IL-17 and IFN-γ played a proinflammatory role in gingival tissues and proresorption role in alveolar bone. However, there might be an antagonistic interaction between IL-17 and IFN-γ. These findings contribute to a better understanding of inflamm-aging cytokines related to alveolar bone resorption in the initiation and progression of PD, thus providing insight into the potential clinical therapeutic targets for PD.

Abbreviations

PD: Periodontal disease
IL-17: Interleukin-17
IFN-γ: Interferon-γ
RANKL: Receptor activator of nuclear factor-κB ligand
TNF-α: Tumor necrosis factor-α
BMM: Bone marrow macrophage
M-CSF: Macrophage colony-stimulating factor
NC: Normal control
NS: Normal saline
HE: Hematoxylin and eosin
TRAP: Tartrate-resistant acid phosphatase
CEJ: Cemento-enamel junction
ABC: Alveolar bone crest
BV/TV: Bone volume per tissue volume
Ctsk: Cathepsin K
NFATc1: Nuclear factor of activated T cells 1
MMP-9: Matrix metallopeptidase 9
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
SASP: Senescence-associated secretory phenotype.

Data Availability

Data will be available from authors on request.

Conflicts of Interest

All authors declare that there are no conflicts of interest associated with this study.

Authors' Contributions

Jingyi Tan and Anna Dai contributed equally to this work.

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Supplementary Materials

Figure S1: the number of TRAP-positive osteoclasts (A) and the area of TRAP-positive osteoclasts (B) were counted and presented (mean ± standard deviation, $n = 3$). $^{\#}p < 0.05$ compared with the control group; $^{\&}p < 0.05$ compared with the IL-17+IFN-γ group. Figure S2: in condition 2, BMMs were cultured with M-CSF (30 ng/ml) for 3 days, and on day 3, 20 ng/ml RANKL together with IL-17 (1 ng/ml) or/and IFN-γ (0.2 ng/ml) was added for 5 days. The number of

TRAP-positive osteoclasts (A) and the area of TRAP-positive osteoclasts (B) were counted and presented (mean ± standard deviation, $n = 3$). $^{\#}p < 0.05$ compared with the control group; $^{\&}p < 0.05$ compared with the IL-17+IFN-γ group. Figure S3: effects of IL-17 (0.1, 10 ng/ml) or/and IFN-γ (0.02, 2 ng/ml) on the expression of osteoclastogenic genes *in vitro*. The mRNA expression of osteoclast-related genes (A) c-Fos, (B) NFATc1, (C) Ctsk, (D) MMP-9, and (E) TRAP was detected with different concentrations of IL-17 or/and IFN-γ using RT-qPCR. Data were standardized to GAPDH expression and shown as a fold change relative to the control group (mean ± standard deviation, $n = 3$). $^{\#}p < 0.05$ compared with the control group; $^{\&}p < 0.05$ compared with the IL-17+IFN-γ group. Table S1: primers used for RT-qPCR. *(Supplementary Materials)*

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Review Article

Complement Inhibitors in Age-Related Macular Degeneration: A Potential Therapeutic Option

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Age-related macular degeneration (AMD) is a multifactorial disease, which can culminate in irreversible vision loss and blindness in elderly. Nowadays, there is a big gap between dry AMD and wet AMD on treatment. Accounting for nearly 90% of AMD, dry AMD still lacks effective treatment. Numerous genetic and molecular researches have confirmed the significant role of the complement system in the pathogenesis of AMD, leading to a deeper exploration of complement inhibitors in the treatment of AMD. To date, at least 14 different complement inhibitors have been or are being explored in AMD in almost 40 clinical trials. While most complement inhibitors fail to treat AMD successfully, two of them are effective in inhibiting the rate of GA progression in phase II clinical trials, and both of them successfully entered phase III trials. Furthermore, recently emerging complement gene therapy and combination therapy also offer new opportunities to treat AMD in the future. In this review, we aim to introduce genetic and molecular associations between the complement system and AMD, provide the updated progress in complement inhibitors in AMD on clinical trials, and discuss the challenges and prospects of complement therapeutic strategies in AMD.

1. Introduction

Age-related macular degeneration (AMD) is a significant cause of irreversible blindness and vision impairment among the elderly in developed countries [1, 2]. With the transformation of the aging population, it is estimated that by 2040, there will be around 288 million AMD patients worldwide [3]. AMD is generally classified into either early, intermediate, or advanced stages, the latter of which can be further subdivided into exudative (wet) and nonexudative (dry) phenotypes [4]. More specifically, wet AMD, also known as neovascular AMD, is characterized by a rapid and substantial vision loss, which is caused by the formation of macular neovascularization. Featuring hemorrhage, edema, and scar formation of retinal tissue, advanced wet AMD occurs partly due to the upregulation of vascular endothelial growth factor (VEGF). Moreover, accounting for about 90% of AMD [5], dry AMD will give rise to macular atrophy and progressive vision loss, featuring photoreceptors, retinal pigment epithelium, and choroidal capillary degeneration, which can be referred to geographic atrophy (GA) as well [6].

The pathogenesis of AMD is complex. Although the identification of genetic risk factors for AMD has been quite successful, the mechanisms by which these risk factors interact in AMD are still unclear. Thus, the discovery of future therapeutic strategy for AMD is still full of challenges. Nowadays, there is still a big gap between dry AMD and wet AMD on treatment. On the one hand, wet AMD is currently treatable. Monthly intravitreal injections of anti-VEGF drugs (such as ranibizumab, aflibercept, and bevacizumab) have become the first-line treatment for wet AMD, which can reduce the incidence of severe vision loss significantly [7]. However, this measure is relatively effective only in a limited population. In this way, a new treatment for wet AMD is also demanded. On the other hand, although some progress has been made in the pathogenesis, there is still no approved

treatment or effective therapy in dry AMD [8]. Consequently, compared with wet AMD, it is more urgent to explore an effective treatment for dry AMD.

Numerous genetic studies and molecular research have confirmed the significant role of the complement system in AMD, including genetic variants, overactivation of alternative pathway, inflammation, oxidative stress, lipid accumulation, and energy metabolism. As the links between the complement system and AMD are becoming clearer, many innovative therapeutic attempts targeted at complement components emerge in the therapy of AMD. To date, there is still a lack of up-to-date and comprehensive papers on the involvement of the complement system in the pathogenesis of AMD and the research actuality of complement inhibitors in the treatment of dry AMD versus wet AMD, even though there have been a number of studies and review articles [9, 10]. This review will focus on the application of complement inhibitors in dry AMD and wet AMD, covering the emerging data on clinical trials, the genetic and molecular associations behind, and the challenges and prospects before this treatment become a formal therapy in the future.

2. Complement System and AMD

2.1. Overview of Complement System. The complement system, first described as an auxiliary system by Jules Bordet and Paul Ehrlich in the late 19th century, is a highly regulated protein network which can be activated in a cascaded manner, and acts at the interface of innate and adaptive immunity [11]. To some extent, the complement system is a double-edged sword. Under normal circumstances, it is the most significant protective mechanism, playing a key role in tissue homeostasis and pathogen immunosurveillance in the body [12]. However, under abnormal circumstances, its impaired regulation or dysfunction can be the main cause of a variety of acute and chronic disorders like atypical hemolytic uremic syndrome, paroxysmal nocturnal hemoglobinuria, C3 glomerulopathy, and AMD.

Generally, the complement cascade is activated via three different proteolytic pathways: the classical pathway (CP), lectin pathway (LP), and alternative pathway (AP) [13] (Figure 1). Specifically, although CP commonly responds to antigen-antibody complexes and LP is started by mannose-binding lectin (MBL) and identification of the polysaccharide or glycoprotein motif on the damaged cell surface [14], both of them will produce a common membrane-bound C4b2a (classical C3 convertase) afterwards. Furthermore, AP can be activated in two ways: by spontaneous hydrolysis of C3 into C3(H_2O) in the fluid phase, also known as "tick-over," and by C4b2a (C3 convertase) to cleave C3 into C3a and C3b in the solid phase [6, 15]. Subsequently, both C3(H_2O) and C3b can be bonded by factor B (FB) and factor D (FD) to form C3(H_2O)Bb and C3bBb, respectively. Of note, AP has a positive feedback on the generation of ample amounts of C3b, which is regarded as the amplification loop between C3bBb and C3b for the other two pathways (Figure 1). Consequently, the two sourced C3 convertases will drive C3b production further in tissues to opsonize the pathogen.

In the final stage, the convergence of the C3 convertase and additional C3b results in the formation of membrane-bound enzyme complexes (C5 convertases), which integrate all processes into a common terminal pathway. The C5 convertase will cleave C5 into C5a and C5b afterwards. Furthermore, owning a new short-lived binding site, C5b sequentially recruits C6, C7, C8, and C9 to form the terminal complex C5b-9, also referred to as the membrane attack complex (MAC). Formation of MAC will lead to a pore in the target cell membrane and ultimately causes cell lysis and death, which can be downregulated by complement inhibitors like CD59 [16, 17].

CD59 is a naturally existing inhibitor of MAC formation that functions by binding to the terminal complement protein complex, preventing C9 molecules from binding to the cell membrane, and thereby forming pores [18]. The complement system can be regulated by different classes of drugs; all levels of the cascade can be affected. Notably, regardless of the initiating pathway, AP accounts for about 80–90% of the activation of the terminal pathway [19]. Thus, overactivation of the complement system in the AP pathway is one of the major drivers of many systemic and organ specific diseases [20, 21].

2.2. AMD as a Multifactorial Disease. As an incredibly complex, multifactorial disease, AMD is driven by a combination of natural aging, unhealthy lifestyle, and genetic predisposition. With increasing age, mitochondria within the RPE cells of AMD patients decrease in size and number and produce more waste products [22, 23], while choroidal elasticity weakens and the ability to process waste products is diminished [24, 25], ultimately leading to impairment of Bruch's membrane function, which triggers a vicious cycle of continuous debris deposition that is considered to be the formation of drusen [26, 27]. In recent years, smoking and nutritional intake have been identified as important risk factors for AMD. The risk of AMD from smoking is dose-dependent, with a reduced risk of developing AMD after quitting, whereas smoking can increase the risk two to fourfold [28, 29]. In terms of diet, a hyperglycemic diet is an important risk factor for AMD [30, 31], whereas a "Mediterranean diet" rich in vitamins and carotenoids may reduce the risk of AMD [32–34]. In addition, fish intake has been shown to have a protective effect against AMD [35]. With regard to genetic variation, genome-wide association studies (GWAS) have established 35 discrete loci with more than fifty independently linked genetic variants, a large proportion of which are related to the complement system [36, 37]. In the following two sections, we will describe the involvement of the complement system in the development and progression of AMD at genetic and molecular levels, respectively.

2.3. Genetic Studies of Complement System in AMD. The relevance between AMD and complement system has been revealed in numerous researches since the 1980s, both locally and systemically: (a) the existence of complement components in drusen, for example, C3a and complement factor H (FH) [27, 38–42]; (b) the elevated levels of systemic [43–46] and local (aqueous humor samples and vitreous

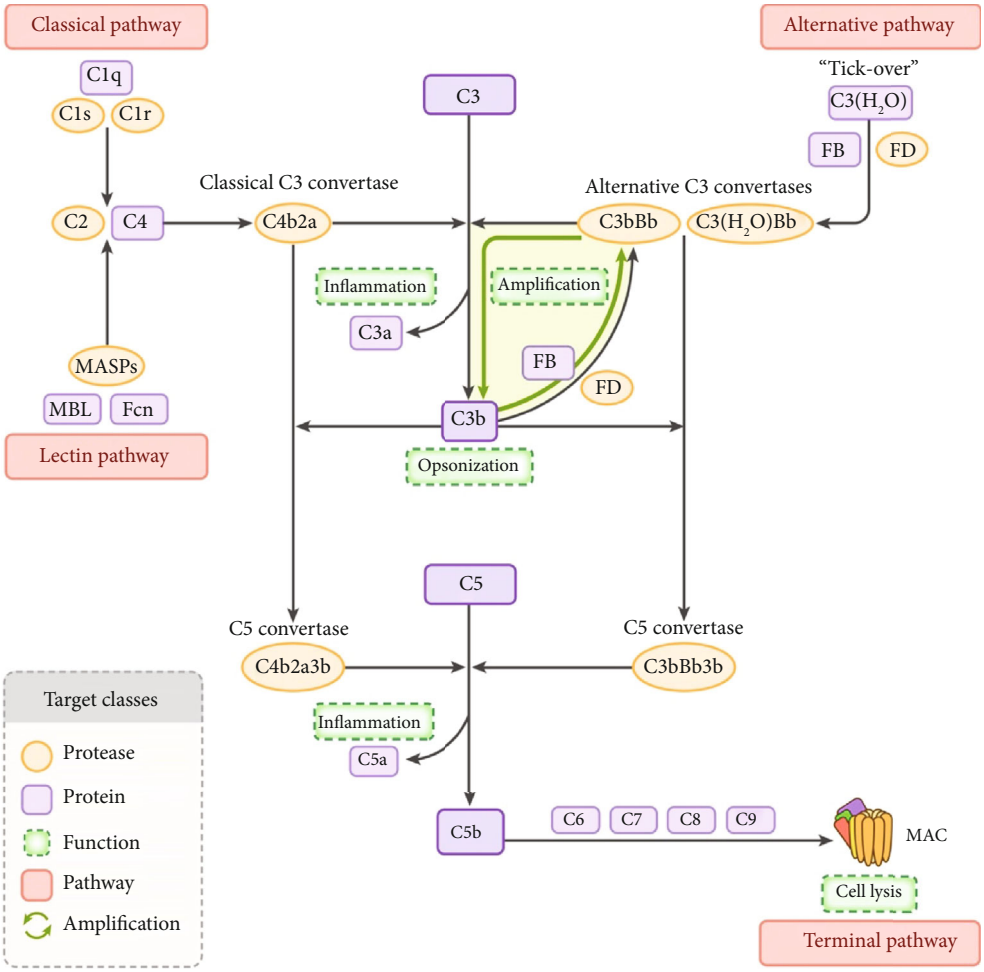

Figure 1: The complement cascade can be activated in three proteolytic pathways: classical pathway (CP), lectin pathway(LP), and alternative pathway (AP). All of them will converge on C3 and C5 and bring about the terminal pathway ultimately, the formation of the membrane attack complex (MAC), which can lead to cell lysis.

humor) complement proteins in AMD patients [20, 47, 48]; (c) the decreased levels of regulatory complement proteins in the eyes of AMD patients [49, 50]; and (d) the increased level of membrane attack complex (a terminal complex of complement cascades) in the retinas of AMD patients [51]. Until 2005, genetic evidence of the complement system in the etiology of AMD was first demonstrated in GWAS [5], which indicated that a common single nucleotide polymorphism (SNP) in the complement FH gene is related to an increased risk of AMD. Subsequently, a lot of studies revealed that other variations in multiple complement genes, such as complement FI, C3, FB, FD, and C9, can increase the risk of AMD as well [37, 52–55].

As an important inhibitor of the complement system, complement FH can not only compete with FB to bind C3b, accelerating the dissociation of the alternative system C3 convertase (C3bBb) resultingly, but also act as a cofactor for facilitating the FI-mediated C3b inactivation [56, 57]. Genetic variations in FH can reduce the effectiveness of modulation in the complement cascade activity, leading to larger precipitation of complement component found in drusen [58]. Up to now, there are a total of 160 coding variants in the FH gene discovered in AMD, among which 16 are nonsense changes, five are frameshift changes, and 139 are missense changes in the FH protein [59]. Nevertheless, their effect needs to be further studied for the vast majority of coding variants in FH. It is worth mentioning that 42% Europeans are heterozygous for the Y402H variant, which is regarded to be consistently associated with the occurrence and progression of AMD. It is reported that homozygous individuals have about 7-fold greater odds of relationship with AMD, while heterozygotes own 2- to 3-fold greater odds of relationship with the disease [15]. The inhibitory effect of FH on the complement system is assumed to be reduced owing to the decreased binding affinity of many complement components of the damaged retina led by the Y402H variant, which further results in excessive chronic local inflammation to occur [49, 60, 61]. Despite extremely rare, R1210C has a closer association with AMD than Y402H [62]. It was presumed that the binding of R1210C mutant to albumin can bring about the loss of function in complement FH [62]. Further studies on functional analysis revealed that R1210C variant decreased binding to C3b, C3d, and heparin to increase activity of the C3 convertase [63–65]. Other rare

variants in complement FH SNPs, including R53C and D90G, have also been noted to influence AMD [62, 66, 67].

As mentioned above, C3 is the key component among all complement pathways. Any functional changes in C3 can directly influence the downriver cascade. Therefore, C3 gene is another important genetic variant in AMD. R102G variant is the most commonly happening mutation in the C3 gene of AMD patients [59]. The polymorphism of R102G in C3 reduces the activity of FH as a cofactor that mediates C3b cleavage by FI. That is, it increases the activation of the AP by prolonging the lifespan of the convertase [68]. Moreover, R80G is a common SNP associated with an ascending risk of AMD [69, 70]. Furthermore, total 91 rare variants in C3 gene are demonstrated in AMD [71, 72]. One of these rare variants is K155Q, positively related to AMD risk, which is located very close to the FH-binding site [73, 74]. Thus, the allele of K155Q can increase the resistance to proteolytic inactivation via FH and FI [75].

The complement FI gene contains a serine protease domain which is the cause of cleaving and inactivating C3b and C4b to regulate complement activation. To date, there are 110 variants in the complement FI gene discovered in AMD patients, among which nine are nonsense changes, one is frameshift change, and 100 are missense changes in the FI protein [59]. A case-control association study for advanced AMD reported that rs10033900A is a common variant near the FI gene, showing the strong association with AMD. A cohort study that included 2,493 advanced AMD patients indicated that 7.8% of AMD cases compared to 2.3% of controls (odds ratio 3.6) are carriers of rare missense changes in the FI variants [73].

Both serine proteases FB and FD have significant roles in the formation and activity of the C3 convertase in AP [6, 76]. Of note, both L9H and R32Q mutations of factor B are considered to have a high protective effect on the development of AMD, although this protective effect may be mediated by FB mutations [77, 78]. Subsequent study has shown that FB fragments are similar to FH levels found in drusen, while R32Q mutations decreased the formation of convertase [79]. Cleaving FB into Ba and Bb fragments, FD is regarded as the rate-limiting enzyme of AP [6, 76]. A small case-control series showed that rs3826945 (a FD gene SNP) is considered positively linked to AMD risk as well [80].

C9 is the most downstream component of the terminal complement pathway. A remarkable decreasing of C9 can affect the production of MAC and finally decrease the cytolytic activity [81–83]. At present, there are 37 variants in the C9 gene discovered in AMD, among which six are nonsense changes, three are frameshift changes, and 28 are missense changes in the C9 protein [59]. The relationship between AMD and a P167S allele in C9 gene has been discovered by genotyping 5,115 independent samples [73]. In another small sample study, the R95X variant in the C9 gene was negatively linked with the risk of AMD [84].

Taken together, on the one hand, these findings suggest that genetic variants that hinder the negative regulation of the complement system promote the development of AMD. Notably, it has been suggested that the additive effect of risk variants leads to an aggregated risk of disease [85]. On the other hand, many variants found in genes related to the complement system highlight the importance of this immunologic pathway in AMD etiology, which can provide potential targets for complement therapy [86–91].

2.4. Molecular Studies of Complement System in AMD. While genetic studies have been somewhat successful in determining the risk of the complement system for the development of AMD, the understanding of how these variants drive AMD at the molecular level remains largely incomplete. Research on the molecular mechanisms of the complement system will not only provide a deeper understanding of AMD but will also contribute to the discovery of future drug treatments. To date, molecular studies of the complement system in AMD have focused on following main areas: chronic inflammation, oxidative stress, lipid accumulation, and energy metabolism.

The most notable consequence of complement activation is that it can mediate the recruitment and activation of immune cells, such as microglia, monocytes/macrophages, lymphocytes, and mast cells, through the release of complement components C3a and C5a [92, 93]. In addition to this, complement activation stimulates surrounding RPE cells to secrete a range of inflammatory factors, for instance, monocyte chemotactic protein 1, interleukin 6, and interleukin 8 [94]. Chronic inflammation is a typical ocular change in AMD. Due to the significant role of the complement system in the inflammatory response to AMD, complement inhibitors targeting C3 and C5 are developed for treatment, and the relevant clinical trials will be described in the next section.

Drusen is a characteristic fundus change in AMD, and it is now known that lipids are a major component of drusen [26]. Indeed, high-risk polymorphisms in the complement gene and dysregulation of the complement system are both related to the local or systemic lipid accumulation. Metabolomic studies performed on plasma/serum from AMD patients have shown a strong correlation between increased high-density lipoprotein (HDL) levels and reduced very low-density lipoprotein (VLDL) and amino acid levels, which are linked with the excessive activation of the complement system. It is now well established that oxidized low-density lipoprotein (LDL) results in the upregulation of C-C motif chemokine receptor 2, interleukin 8, tumor necrosis factor, and VEGF and that binding of FH to these oxidized LDLs attenuates inflammation [95–97]. However, it remains unknown whether the presence of FH in HDL particles has a protective effect [21].

In recent years, several studies have exposed RPE cells to different types of stress stimuli separately, measuring and analyzing the levels of complement proteins and complement factors. For example, extracellular levels and genetic levels of C3 were significantly improved when human adult retinal pigment epithelial cells (ARPE-19) were exposed to H_2O_2, smoke extracts, and lipid oxidation products [98–102]. In addition, FH has been found to in human plasma in two distinct redox forms. The reduced form is higher in patients with early AMD and protects ARPE19 cells from oxidative damage, whereas the oxidized form is higher in patients with advanced AMD and can effectively mediate FI to accelerate C3 cleavage [103].

Recently, imbalances in energy metabolism within RPE cells have been considered as an important aspect of AMD pathology. An experimental animal study showed abnormally large mitochondria, reduced levels of mitochondrial DNA, and decreased ATP production in photoreceptors and RPE cells in FH knockout mice [104, 105]. This suggests that dysregulation of the complement system can reduce cellular energy metabolism by affecting the structure and function of mitochondria. Furthermore, a link between the complement system and autophagy-lysosomes has recently been demonstrated [106].

3. Complement Inhibitors and AMD

3.1. Overview of Complement Therapy in AMD. The current treatment strategy for AMD is not optimistic. Although anti-VEGF injection is an effective treatment option for wet AMD, there is still a proportion of patients who are not sensitive to the drug. In addition, even with monthly anti-VEGF therapy, GA still remains an inevitable long-term progressive outcome for a majority of patients with wet AMD [107, 108]. However, there are no approved treatments or effective approaches for dry AMD. Therefore, the exploration of treatments for AMD remains an important area of research for ophthalmologists.

In the past decades, as the first FDA-approved complement inhibitor, eculizumab (C5 inhibitor) is used to treat the hemolytic disorder, paroxysmal nocturnal hemoglobinuria (PNH), which remarks an important milestone in complement drug discovery [109]. Since then, complement inhibitors have been receiving more attention gradually. To date, more than 14 complement inhibitors have been generated for core complement components (C3, C5) and complement regulators (FD, FI, etc.), and a total of nearly 40 clinical trials of these complement inhibitors have been completed or are under way in AMD. The main outcome indicators in these clinical trials are changes in the size of the GA lesion or improvements in visual function. Given the urgent need for the treatment of dry AMD, the majority of these clinical trials have focused on GA [87]. Here, we will classify the clinical trials according to dry AMD and wet AMD and update the progress of them, respectively.

3.2. Complement Inhibitors in Dry AMD. Although numerous studies have explored the etiology and pathogenesis of dry AMD, there is still a lack of an effective strategy for the treatment. Therefore, compared with wet AMD, it is more urgent to devise an efficiently and feasible treatment for dry AMD. According to the statistics, in dry AMD, there are at least 13 different complement inhibitors which have been applied in nearly 30 different clinical trials (Table 1). Next, we will summarize them one by one according to the common targets of different drugs.

Targeting at C3, three therapeutic drugs have been devised for dry AMD so far. Firstly, POT-4, also referred to as AL-78898A, is a compstatin derivative, which is also the first complement inhibitor to be employed in patients. Preliminary results have shown that intravitreal POT-4 is safe and well-tolerated (NCT00473928). However, the trial was terminated prematurely due to the deposit formation of drugs in the eye of GA in phase II trial (NCT01603043). The second is APL-2, also known as pegcetacoplan, another peptide compstatin analogue, which reduces the GA growth rate by 29% monthly and by 20% every other month (EOM) in phase II trial, compared with the sham treatment group (NCT02503332) [110]. Of note, the neovascularization of dry AMD was reported more frequently in APL-2-treated eyes (20.9%, 8.9%, and 1.2% in monthly groups, EOM groups, and sham groups, respectively). Phase III clinical trials of APL-2 are under way with the same dosage regimen, but the difference is that the duration of treatment has been extended to 2 years (NCT03525613). Moreover, an extension study to assess the long-term safety and efficacy is currently under way (NCT03777332). Lastly, as a humanized IgG1 monoclonal antibody engineered to potently inhibit the activity of C3, the performance of NGM621 is safe and well-tolerated in phase I trials. Further studies will be conducted with doses of 15 mg injected every 4 weeks or every 8 weeks in phase II trial (NCT04465955).

Targeting at C5, there are three therapeutic drugs explored for GA up to now. First, as the first FDA-approved complement inhibitor, eculizumab was safe and well-tolerated in the body through 6 months. However, eculizumab did not significantly reduce the growth rate of GA or the volume of drusen by intravenous fluid (NCT00935883) [111, 112]. Secondly, as a monoclonal C5 inhibitor, LFG316 (tesidolumab) has passed the safety evaluation but still inhibits the progression of GA lesions ineffectively in phase II trials (NCT01527500). Finally, Zimura, also known as avacincaptad pegol, is a pegylated RNA aptamer, which is a specific and potent inhibitor of C5. Compared with the sham group, intravitreal administration of Zimura 2 mg and 4 mg dose groups can reduce 27.4% and 27.8% of mean rate of GA growth over one year, respectively, which is of statistical significance in the phase II/III trials (NCT02686658) [113]. In addition, treatment with Zimura showed the increased dose-dependent risk of CNV in treated eyes: 2.7%, 11.9%, and 15.7% in sham, 2 mg dose, and 4 mg dose cohorts, respectively. Currently, focusing on the safety and efficacy of Zimura in slowing down the rate of GA growth, phase III trials of confirmation are under way (NCT04435366).

On the one hand, several inhibiting activators have been targeted in clinical trials, such as FD, properdin, and FB. As is known to all, complement FD is a pivotal activator of complement AP, which is the target of lampalizumab, a selective monoclonal complement FD inhibitor. In phase II, a positive result indicated that monthly lampalizumab treatment demonstrates a 20% reduction in the progression of GA lesion area (NCT02288559) [114]. However, the greatest studies of GA have shown that lampalizumab does not decrease the enlargement of GA over 12 months in phase III, and this trial terminated afterwards (NCT02247479, NCT02247531) [115, 116]. On top of that, CLG561, a fully human antibody Fab, can neutralize properdin to prevent the formation of early and late activation products. In phase I trials, single intravitreal doses of CLG561 are safe and well-tolerated (NCT01835015). Unfortunately, CLG561 were evaluated as a monotherapy or in combination with LFG316 (a C5

Table 1: Summary of complement inhibitors in clinical trials for dry AMD.

Target	Drug (sponsor)	Administration	Phase	Design	Sample size (n=)	Primary outcome measure	Status	First posted	Trial number	Clinical outcome
C3	POT-4 (Alcon)	Intravitreal	II	Monthly vs. sham	10	GA area change at month 12 by FAF	Terminated	2012	NCT01603043	Drug deposit formation
	APL-2 (Apellis)	Intravitreal	II	Monthly vs. EOM vs. sham	246	Square root of GA area change at 12 months	Completed	2015	NCT02503332	29% reduction in GA lesion growth
	APL-2 (Apellis)	Intravitreal	III	Monthly vs. EOM vs. sham	600 (estimated)	GA area change at month 12 by FAF	Ongoing	2018	NCT03525613	—
	APL-2 (Apellis)	Intravitreal	III	Monthly vs. EOM vs. sham	1200 (estimated)	Percentage of adverse events at month 36	Ongoing	2021	NCT03777332	—
C5	NGM621 (NGM bio.)	Intravitreal	II	Every 4 weeks vs. 8 weeks vs. sham	240 (estimated)	GA area change at week 48 by FAF	Ongoing	2020	NCT04465955	—
	Eculizumab (Alexion)	Intravenous	II	Low dose vs. high dose	60	Growth of GA and decrease in drusen volume	Completed	2009	NCT00935883	Lack of efficacy
	LFG316 (Novartis)	Intravitreal	I	SAD	24	Safety and tolerability	Completed	2010	NCT01255462	Safe and well-tolerated
	LFG316 (Novartis)	Intravitreal	II	Low dose vs. high dose vs. sham	158	GA area change at day 505 by FAF	Completed	2012	NCT01527500	Lack of efficacy
	ARC1905 (Ophthotech)	Intravitreal	I	Low dose vs. high dose	47	Safety and tolerability	Completed	2009	NCT00950638	Safe and well-tolerated
	ARC1905 (Ophthotech)	Intravitreal	II/III	Low dose vs. high dose vs. sham	286	GA area change at month 12 by FAF	Completed	2016	NCT02686658	27% reduction in GA lesion growth
	ARC1905 (Ophthotech)	Intravitreal	III	Monthly vs. EOM vs. sham	400 (estimated)	Mean rate of GA change at month 12 by FAF	Ongoing	2020	NCT04435366	—
FD	Lampalizumab (Roche)	Intravitreal	Ia	SAD	18	Safety and tolerability	Completed	2009	NCT00973011	Safe and well-tolerated
	Lampalizumab (Roche)	Intravitreal	II	Every 2 weeks vs. 4 weeks vs. sham	96	GA area change at week 24 by FAF	Completed	2014	NCT02288559	20% reduction in GA lesion area
	Lampalizumab (Roche)	Intravitreal	II	Monthly vs. EOM vs. sham	159	Percentage of adverse events	Terminated	2012	NCT01229215 NCT01602120	Lack of efficacy
	Lampalizumab (Roche)	Intravitreal	III	Every 4 weeks vs. 6 weeks vs. sham	906	GA area change at week 48 by FAF	Terminated	2014	NCT02247479 NCT02247531	Lack of efficacy
	Lampalizumab (Roche)	Intravitreal	III	Every 4 weeks vs. every 6 weeks	994	Percentage of adverse events at week 96	Terminated	2016	NCT02745119	Lack of efficacy
Properdin	CLG561 (Novartis)	Intravitreal	I	SAD (5 dose levels)	50	Safety and tolerability	Completed	2013	NCT01835015	Safe and well-tolerated
	CLG561 (Novartis)	Intravitreal	II	CLG561 vs. CLG561 +LFG316 vs. sham	114	Percentage of adverse events, GA area change at day 337 by FAF	Completed	2015	NCT02515942	Lack of efficacy

Table 1: Continued.

Target	Drug (sponsor)	Administration	Phase	Design	Sample size (n=)	Primary outcome measure	Status	First posted	Trial number	Clinical outcome
FB	IONIS-FB-LRx (Lonis)	Subcutaneous	II	IONIS-FB-LRx vs. placebo	330 (estimated)	GA area change at week 49 by retinal imaging	Ongoing	2019	NCT03815825	—
CD59	AAVCAGsCD59 (Hemera)	Intravitreal	I	SAD (3 dose levels)	17	Percentage of adverse events at week 26	Completed	2017	NCT03144999	Safe and well-tolerated
	AAVCAGsCD59 (Hemera)	Intravitreal	II	Low dose vs. high dose vs. sham	132 (estimated)	GA area change at month 24 by FAF	Ongoing	2020	NCT04358471	—
FI	GT005 (gyroscope)	Subretinal	I/II	SAD (3 dose levels)	45 (estimated)	Percentage of adverse events at week 48	Ongoing	2019	NCT03846193	—
	GT005 (gyroscope)	Subretinal	II	Low dose vs. high dose vs. sham	180 (estimated)	GA area change at week 48 by FAF	Ongoing	2020	NCT04437368 NCT04566445	—
FH	GEM103 (Gemini)	Intravitreal	I	SAD (4 dose levels)	12	Safety and tolerability	Completed	2020	NCT04246866	Safe and well-tolerated
	GEM103 (Gemini)	Intravitreal	II	GEM103 vs. sham	45 (estimated)	Percentage of adverse events at month 18	Ongoing	2020	NCT04643886	—
C1q	ANX007 (Annexon)	Intravitreal	II	Monthly vs. EOM vs. sham	240 (estimated)	GA area change at month 12 by FAF	Ongoing	2020	NCT04656561	—

FD: factor D; FB: factor B; FI: factor I; FH: factor H; EOM: every other month; SAD: single ascending dose; GA: geographic atrophy; FAF: fundus autofluorescence.

inhibitor), and there is no effect on the change of GA lesions in the phase II clinical trial (NCT02515942). Besides, as a novel drug, IONIS-FB-LRx is a ligand-conjugated antisense inhibitor, which can be administered subcutaneously for systemic reduction in circulating FB levels, with a potential to diminish the systemic overactivity of the alternative pathway. It is being assessed in a phase II trial for dry AMD patients. In phase I trials, subcutaneous injection of IONIS-FB-LRx has been proved safe in 54 healthy volunteers. Based on this, a phase II trial has been initiated to evaluate the effectiveness of IONIS-FB-LRx in GA patients (NCT03815825).

On the other hand, a few supplementing regulators of the complement system have been targeted in clinical trials, for example, CD59, FI, and FH. Firstly, AAVCAGsCD59, also named as HMR59, uses an AAV2 gene therapy and is designed to induce the generation of a soluble CD59 protein, which binds the incomplete MAC and blocks the binding of the C9 protein required to complete the complex [117, 118]. In phase I clinical trials, AAVCAGsCD59 is delivered intravitreally and well-tolerated (NCT03144999). Of note, while the phase I trials are not designed to judge efficacy, it is encouraging that 9 of 11 cases demonstrated a slower rate of GA growth, which promotes it to be tested for the efficacy of GA in phase II trials (NCT04358471).

Furthermore, two clinical trials based on genotype to select patients have been performed. Specifically, before the inclusion in the trials of GT005 and GEM103, patients are chosen for carrying risk gene variants in complement FI and FH. Similar to AAVCAGsCD59, GT005 uses an AAV vector and is designed to the supply of FI protein to the treated eye. Nowadays, phase I/II trials of GT005 in patients with GA are assessing its effectiveness in the UK. As therapy via subretinal injection, GT005 is currently in phase II clinical trials (NCT03846193). Moreover, gene therapy provides the potential for a single injection lasting for 1 year or longer, which offers the benefit of requiring fewer injections to treat AMD patients should the drug show efficacy and safety in future trials. As an endogenous human FH protein, GEM103 is administered via intravitreal injection. The safety and tolerability of GEM103 has been shown in phase I trials. Ocular pharmacokinetic and pharmacodynamic effects were assessed in aqueous humor samples for two months after treatment, showing that sustained supranormal levels of drug concentrations were achieved after treatment.

Finally, ANX007 is a monoclonal antibody antigen-binding fragment (Fab), which can potently bind to C1q to inhibit the activation of CP, including C3 and C5. ANX007 has been tested in patients with primary open-angle glaucoma, and the safety is confirmed, and it is well-tolerated in the eye. At present, ANX007 is used in investigating the safety and efficacy of intravitreal injections in patients with GA in phase II (NCT04656561).

3.3. Complement Inhibitors in Wet AMD. Since the approval of the first injectable anti-VEGF drug in 2004, intravitreal injections of anti-VEGF drugs have become the first-line treatment for wet AMD patients to suppress CNV and improve visional function currently [119]. A recent systematic literature review demonstrated that the introduction of anti-VEGF therapy in clinical practice has been associated with a significant reduction in the prevalence of blindness [120]. Nevertheless, this strategy still has a few limitations. Rofagha et al. studied the outcome of about 7-year ranibizumab-treated patients and found only one-third of the outcomes to be good with a visual decline observed in half of the patients [121]. Thus, there are still vast unmet clinical demands in wet AMD; exploring new treatment strategies is still needed. Up to now, there are 6 complement inhibitors which have been investigated in 10 clinical trials in wet AMD (Table 2).

Targeting at C3, two therapeutic drugs have been devised for wet AMD patients so far. The intravitreal delivery of POT-4 was compared to anti-VEGF in patients with active wet AMD in a phase II trial. As a result, the advantage of POT-4 over anti-VEGF drugs could not be demonstrated. Instead, the AMD participants treated with POT-4 had increased retinal thickness at week 4, while patients injected with anti-VEGF had decreased retinal thickness. POT-4 was unable to reduce central retinal thickness 12-week posttreatment, as seen with ranibizumab (NCT01157065). The other is APL-2, which is delivered intravitreally and well-tolerated in phase I trials (NCT02461771). APL-2 were evaluated in patients with active wet AMD on anti-VEGF treatment in phase II as well. However, this clinical trial was terminated due to the ineffectiveness of treatment when 17 participants were collected (NCT03465709).

Targeting at C5, there are two therapeutic agents that have been explored in wet AMD to date. LFG316 drug was also assessed for efficacy in phase II trials, unfortunately, neither reduction in the number of anti-VEGF injections (NCT01535950) nor improvement in BCVA and macular thickness (NCT01624636). Zimura combination therapy with ranibizumab for wet AMD was safe and well-tolerated after 6 months of treatment in a phase II trial as well (NCT00709527, NCT03362190). Of note, in patients receiving monthly Zimura 2 mg in combination with anti-VEGF 0.5 mg, approximately 60% achieved visual acuity improvement greater than or equal to three lines, which was better than the results of anti-VEGF monotherapy.

Moreover, two new drugs are undergoing phase I clinical trials. Firstly, intravitreal delivery of AAVCAGsCD59 is currently in phase I clinical evaluation (NCT03585556). Previous studies have shown that subretinal injection of AAVCAGsCD59 attenuated the formation of laser-induced CNV by around 60% in mice [122]. In phase I trials, all new-onset wet AMD patients received anti-VEGF treatment at day 0 and subsequently accepted intravitreal AAVCAGsCD59 for one week. The combination of gene therapy and anti-VEGF treatment is regarded as a promising approach to wet AMD patients [123]. The other is IBI302, which is globally the first bispecific recombinant fully human fusion protein. Its N-terminus binds to VEGF family, thereby blocking the VEGF-mediated signaling pathway. Its C-terminus binds specifically to C3b and C4b, inhibiting the activation of the complement cascade of CP and AP and reducing the complement-mediated inflammatory response, leading to the treatment of wet AMD. The additional targets added to IBI302 could provide larger clinical benefit

Table 2: Summary of complement inhibitors in clinical trials for wet AMD.

Target	Drug (sponsor)	Administration	Phase	Design	Sample size (n=)	Outcome measure	Status	First posted	Trial number	Clinical outcome
C3	POT-4 (Potentia)	Intravitreal	I	SAD	27	Safety and tolerability	Completed	2007	NCT00473928	Safe and well-tolerated
	POT-4 (Potentia)	Intravitreal	II	POT-4 vs. ranibizumab	99	Central subfield retinal thickness at week 4	Completed	2010	NCT01157065	Lack of efficacy
	APL-2 (Apellis)	Intravitreal	I	SAD	13	Safety and tolerability	Completed	2015	NCT02461771	Safe and well-tolerated
C5	APL-2 (Apellis)	Intravitreal	Ib/II	APL-2	17	Percentage of adverse events at 1 year	Terminated	2018	NCT03465709	Lack of efficacy
	LFG316 (Novartis)	Intravitreal	II	LFG316 vs. sham	43	Number of anti-VEGF treatments	Completed	2012	NCT01535950	Lack of efficacy
	LFG316 (Novartis)	Intravenous	II	Low dose vs. high dose vs. placebo	1	Safety and tolerability	Terminated	2012	NCT01624636	Lack of efficacy
	ARC1905 (Ophthotech)	Intravitreal	I	ARC1905+ranibizumab vs. ranibizumab	60	Safety and tolerability	Completed	2008	NCT00709527	Safe and well-tolerated
	ARC1905 (Ophthotech)	Intravitreal	IIa	ARC1905 (3 dose levels)+ranibizumab	65	Percentage of adverse events at month 6	Completed	2017	NCT03362190	No adverse events
CD59	AAVCAGsCD59 (Hemera)	Intravitreal	I	AAVCAGsCD59+anti-VEGF	25 (estimated)	Number of anti-VEGF treatments	Ongoing	2018	NCT03585556	—
C3b C4b	IBI302 (Innovent)	Intravitreal	I	IBI302 (6 dose levels)	180 (estimated)	Safety and tolerability	Ongoing	2019	NCT03814291	—

compared to anti-VEGF drugs. The study was presented at the 2020 American Academy of Ophthalmology (AAO). To date, 31 subjects have participated in phase I clinical trials, and the safety and tolerability of IBI302 are well (NCT03814291). The results from these clinical trials will provide insight into the potential of complement inhibition and combination therapy in the future [124].

4. Challenges and Prospects

Nowadays, despite complement inhibitors seeming promising in theory or even in vitro studies, the majority of clinical trials aiming at this class of drugs in AMD prove to have a modest effect, which might be relevant to the following five reasons.

First of all, the selective permeability of drugs may be a factor that cannot be ignored. Indeed, our eye is a highly protected organ and the blood-ocular barrier effectively shields it from macromolecules. In general, the molecule cannot penetrate the eye when its size exceeds 76.5 ± 1.5 kDa [125]. FD has been shown to diffuse through Bruch's membrane, whereas most of the complement components do not, such as FH, FI, FB, and C3/C3b [126]. Thus, it has been speculated that the failure of eculizumab is because of its large molecular size (148 kDa) [127], and the results of this trial might have been altered with better drug design and improvements in the drug delivery system.

Secondly, the timing of intervention may be an important reason. Almost all complement inhibitors focus on the treatment of advanced stage in dry AMD, but the results are barely succeeded. A possible explanation may be that when the disease has evolved to the stage of GA, irreversible structural and functional damage of the retina has already occurred at this point [128]. Not surprisingly, even though the complement system is the significant factor in the pathogenesis of AMD, the clinical treatment efficacy of complement inhibitors is likely to be minimal at that time. Consequently, it may be a new breakthrough point to initiate the intervention at an earlier stage in the following study [127].

Thirdly, the inconsistency between the target of drug therapy and the dominant cause of patients may be another significant factor. Given that AMD is a multifactorial disorder, complement inhibitors that act on a specific target may be only suitable for patients who carry a genetic risk factor predisposing to overactivation of complement at that specific target, while not applied to all AMD patients. For instance, patients who are homozygous for the FH 402H variant would have a 7.4-fold greater risk from AMD in elderly people than those who are heterozygous for the 402H variant [53]. Therefore, correct selection of patients who have genetically supported targets to conduct drug development accordingly and clinical trials may increase the odds of success in clinical drug development [129].

Fourthly, the frequency of intervention might be a potential reason as well. For example, while lampalizumab demonstrated the potential to improve the progression of GA in phase II trials, it was unable to reproduce such outcome and efficacy in phase III trials. A probable cause may be the lower frequency of medical treatment, every 2 or 4 weeks in phase II while every 4 or 6 weeks in phase III, reducing the effectiveness of treatment [115, 130]. In this way, the medical influence brought by different frequencies of complement therapy is of significant value and deserves to be studied further in the future.

Finally, the limitations of assessment methods may also be a relevant factor. At present, in most clinical trials, the checking means of evaluating the curative effect is fundus autofluorescence (FAF). However, FAF has poor susceptibility to media opacities because of the macular pigment that absorbs blue light, thereby causing difficulty in imaging the fovea [131]. Therefore, the accuracy of the efficacy assessment for those patients will also be affected. Moreover, quantitative evaluation of efficacy remains to be a challenge [81]. If more accurate and sophisticated detection tools could be devised, then the effects of complement inhibitors on patients with AMD can be observed more creditably and comprehensively in future studies.

Although complement therapy is confronted with multiple challenges currently, its therapeutic future remains promising. Both APL-2 and Zimura have demonstrated modest success in inhibiting the progress of GA. With the improvement of visual acuity as an ideal goal, both of them have passed phase II clinical trials and are undergoing further verification in phase III clinical trials. Of note, the inhibition of C3 through APL-2 induced neovascularization, prompting a tendency to convert dry AMD to wet AMD. Even though there remain unrevealed risks of neovascularization in dry AMD, treatment with anti-VEGF after conversion to wet AMD is also a possible treatment strategy for this group of patients [81]. In addition, the performance of several new drugs and combination therapies, such as AAVCAGsCD59, applied in phase II trials is also promising.

5. Conclusion

The complement system indeed plays a remarkable role in the development and progression of AMD. Targeting at different complement components, many clinical trials of complement inhibitors have been conducted or are ongoing in AMD. While most complement inhibitors fail to demonstrate the potential, APL-2 and Zimura are effective in inhibiting the rate of GA progression in phase II clinical trials, and both of them successfully entered phase III trials. Furthermore, the combination of Zimura and ranibizumab resulted in a significant improvement in visual acuity in patients with wet AMD at phase II clinical trials compared to ranibizumab monotherapy. Of note, the performance of Zimura in both dry AMD and wet AMD is quite encouraging. However, whether Zimura is expected to be a new drug for dry AMD is pending the results of phase III clinical trials. Overall, complement inhibitors have shown potential in the future treatment of AMD and deserve to be explored further.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors' Contributions

Xue Feng and Yanling Wang share the correspondence authorship of the manuscript.

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Review Article

The Roles of FOXO1 in Periodontal Homeostasis and Disease

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Periodontitis is an oral chronic inflammatory disease that is initiated by periodontal microbial communities and requires disruption of the homeostatic responses. The prevalence of periodontal disease increases with age; more than 70% of adults 65 years and older have periodontal disease. A pathogenic microbial community is required for initiating periodontal disease. Dysbiotic immune-inflammatory response and bone remodeling are characteristics of periodontitis. The transcription factor forkhead box protein O1 (FOXO1) is a key regulator of a number of cellular processes, including cell survival and differentiation, immune status, reactive oxygen species (ROS) scavenging, and apoptosis. Although accumulating evidence indicates that FOXO1 activity can be induced by periodontal pathogens, the roles of FOXO1 in periodontal homeostasis and disease have not been well documented. The present review summarizes how the FOXO1 signaling axis can regulate periodontal bacteria-epithelial interactions, immune-inflammatory response, bone remodeling, and wound healing.

1. Introduction

Periodontitis is a chronic inflammatory disease of the tooth-supporting tissues that is initiated by pathogenic microbial communities and results in progressive destruction of the periodontal tissues, including the gingiva, periodontal ligaments, and alveolar bone [1, 2]. Severe periodontitis is the 6th most prevalent disease worldwide [3]. Epidemiological evidence shows that the occurrence and severity of periodontitis increase with age [4]. While early studies indicated that a triadic group of microbes comprising *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* was the causative agent of periodontitis, more recent microbiome studies have discovered greater complexity to the etiology of periodontitis [5]. Emerging data show that complex microbial communities are the fundamental etiological agent, and periodontitis results from polymicrobial synergy within these communities which incites dysbiotic host responses [6, 7]. Colonization by keystone pathogens (e.g., *P. gingivalis*) can initiate a transition of the periodontal microbial community from a commensal microbiota to a dysbiotic microbiota, triggering host immune responses and facilitating pathobiont persistence in the local environment, further dysregulating the host immune-inflammatory state [1, 2]. Moreover, microbial dysbiosis and inflammation can reinforce each other in a reciprocating feedforward loop, leading to periodontal tissue breakdown [8]. For instance, colonization by *P. gingivalis* stimulates host cells to release various proinflammatory cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), and recruits neutrophils to the site of infection [9]. Neutrophils can also induce the generation of ROS via the respiratory burst. At low concentrations, ROS are part of the host defense against infection [10]. Tamaki et al. found that the levels of reactive oxygen metabolites in the serum were positively correlated with immunoglobulin G antibodies against specific periodontal pathogens, including *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Prevotella intermedia* [11].

Although a dysbiotic microbial community is required for initiation of periodontal disease, it should be noted that the deleterious effects of the host response to the microbial challenge, rather than the direct toxic role of microbiota, are the main cause of periodontal damage [8]. The gingival epithelium that lines the gingival crevice forms a barrier between colonizing bacteria and gingival tissues and together with antimicrobial proteins, e.g., defensins and antimicrobial

peptides (AMPs), provides the first line of defense against invading periodontal pathogens [12]. Once this barrier is overcome, periodontal tissue destruction and bone resorption are the primary outcome of interactions between the microbiota and immune cells, including phagocytes, natural killer (NK) cells, dendritic cells (DCs), T cells, and B cells [13]. The cytokine system is a key modulator in the process. For instance, the well-established proinflammatory cytokines from IL-1, IL-6, and TNF families can exaggerate periodontal inflammatory responses and lead to tissue degradation. A persisting inflammatory environment may ultimately disrupt bone homeostasis. In particular, cytokines such as IL-1β, TNF, and IL-17 can stimulate the expression of the receptor of nuclear factor-κB ligand (RANKL), thus inducing the maturation and activation of osteoclasts [14]. Therefore, a homeostatic balance between immune-inflammatory responses and antimicrobial activities as well as a balance between osteoblasts and osteoclasts is required for periodontal health. Numerous clinical periodontal reconstructive surgeries have been attempted to restore such lost tissues [15], and these surgical procedures can lead to different patterns of healing. Nevertheless, epithelization of the wound and wound stability are indispensable for the establishment of a new connective tissue attachment to a root surface [16].

The forkhead box O (FOXO) transcription factors regulate many facets of the cellular physiological process, such as oxidative stress response, apoptosis, cell cycle regulation, and cell survival and differentiation [17]. The FOXO family has four members in humans, including FOXO1, FOXO3, FOXO4, and FOXO6. FOXO1 is normally expressed in insulin-responsive tissues and organs, such as the liver, skeletal muscle, and adipose tissue [18], and has been extensively studied since it was first identified in alveolar rhabdomyosarcomas [19]. A potential role for FOXO1 in periodontal homeostasis and dysbiosis is emerging. To contribute to the understanding of this issue, the present review focuses on the involvement of FOXO1 in regulating periodontal bacteria-epithelial interactions, immune response, bone remodeling, and wound healing.

2. Regulation of FOXO1 Activity and Expression

FOXO1 is considered a master control switch for multiple signals that enable an organism to maintain tissue homeostasis during stress [20]. The transcriptional activity of FOXO1 is regulated through a number of posttranslational modifications (PTMs), including phosphorylation, acetylation, ubiquitination, methylation, and O-GlcNAcylation. These PTMs affect FOXO1 subcellular localization, stability, and activity as a transcriptional regulator. FOXO1 along with other forkhead box O transcription factors (FOXO3, FOXO4, and FOXO6) shares a highly conserved 110-amino-acid DNA-binding domain, also known as the forkhead box or winged helix domain. These proteins also share a compact α/β fold that consistently contains four α helices (H1-H4), three β strands (S1-S3), and two wings (W1 and W2) (Figure 1). The regions showing the highest sequence conservation include the N-terminal region surrounding the first AKT/protein kinase (PKB) phosphorylation site, the forkhead DNA-binding domain (DBD), the region containing the nuclear localization signal (NLS), and part of the C-terminal transactivation domain [21]. Several sites for posttranslational modifications are located within or near the FOXO DBD, thus enabling the regulation of the interaction of FOXO with DNA, either directly or through protein-protein interactions [21–23].

Shuttling of FOXO1 between the nucleus and cytoplasm requires protein phosphorylation within several domains, and these are regulated by distinct signal transduction pathways, including the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway, the mitogen-activated protein kinase/extracellular regulated protein kinase (MAPK/ERK) pathway, and the c-Jun N-terminal kinase (JNK) pathway [24, 25]. Table 1 provides a summary of known FOXO1 phosphorylation sites. Active PKB translocates to the nucleus and phosphorylates FOXO1 at three conserved residues, resulting in increased binding of FOXO1 to the regulator 14-3-3 and cytoplasmic localization of both proteins [20, 26]. Following cellular stress, particularly when high levels of ROS are generated, JNK becomes activated and phosphorylates FOXO1. This causes FOXO1 to translocate in the opposite direction, enter the nucleus, and display increased transcriptional activity [27].

Acetylation has also been demonstrated to regulate FOXO1 activity. The cyclic-AMP responsive element binding- (CREB-) binding protein (CBP), histone acetyltransferase paralogue p300, and p300/CBP-associated factor (PCAF) can acetylate lysine residues located in the forkhead domain [28, 29]. Conversely, enzymes of the sirtuin (Sirt) family catalyse NAD^+-dependent deacetylation of FOXO1. Seven lysine residues (K245, K248, K262, K265, K274, K294, and K559) have been established as acetylation sites in FOXO1 [22, 28]. The acetylation of FOXO1 has been shown to result in both activation and inhibition of its transcriptional activity, depending on the cell types used and the FOXO1 target genes [30–32]. In most studies, deacetylation contributes to elevated FOXO1 activity and its transduction from the cytoplasm to the nucleus [31]. In addition, the above-mentioned lysine residues in FOXO1 can be ubiquitinated by S-phase kinase-associated protein 2 (Skp2).

The expression of FOXO1 genes is regulated in response to multiple physiological cues and pathological stimuli, such as oxidative stress and hormonal factors. E2 promoter binding factor 1 (E2F-1), p300, and forkhead box protein C1 (FOXC1) play critical roles in regulating FOXO1 gene transcription [33]. Additionally, numerous microRNAs and the RNA-binding protein, HuR, have been described as posttranscriptional regulation mechanisms of FOXO1 [34].

3. The Role of FOXO1 in Periodontal Homeostasis and Disease

As a critical signaling integrator, activated FOXO1 participates in maintenance of homeostasis and adaptation to environmental changes, properties that are important in periodontal health. Disruption of physiologic FOXO1 signaling, therefore, has potential relevance for periodontal dysbiosis.

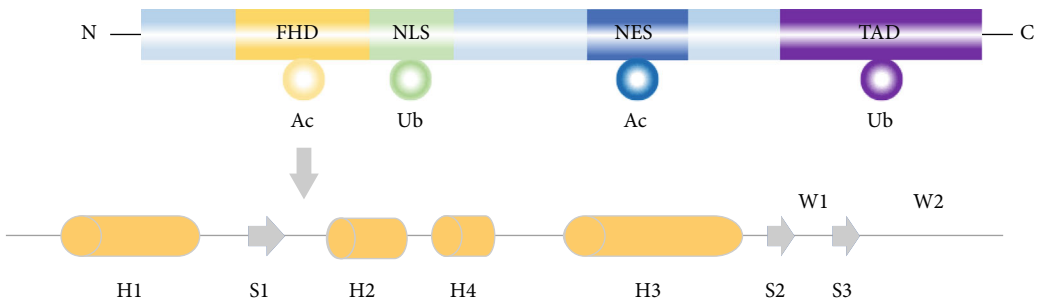

Figure 1: The structure of FOXO1. From the N terminus to the C terminus, FOXO1 contains a forkhead DNA-binding domain (FHD), a nuclear localization signal (NLS), a nuclear export sequence (NES), and a transactivation domain (TAD). Ac: acetylation; Ub: ubiquitination. A compact α/β fold contains four α helices (H1-H4), three β strands (S1-S3), and two wings (W1 and W2).

Table 1: Phosphorylation sites in FOXO1 protein.

Kinases	Abbreviations	Sites phosphorylated in FOXO1	The role on FOXO1 activity	References
Protein kinase B	AKT	T24, S256, S319	Inactivation, nuclear exclusion	Guo et al. (1999); Rena et al. (1999)
c-Jun N-terminal kinase	JNK	S256	Activation, nuclear localization	Wang et al. [37]
Extracellular regulated protein kinase	ERK	S246, S284, S295, S326, S413, S415, S429, S467, S475	Enhanced interaction with other transcription factors suggested	Asada et al. (2007)
p38 mitogen-activated protein kinase	p38MAPK	S284, S295, S326, S467, S475	Enhanced interaction with other transcription factors hypothesized	Asada et al. (2007)
Cyclin-dependent kinase 1	CDK1	S249	Activation, nuclear localization	Yuan et al. (2008)
Cyclin-dependent kinase 2	CDK2	S249	Inactivation, nuclear exclusion (S249 phosphorylation verified, but no nuclear exclusion in some cells)	Huang et al. (2006); Yuan et al. (2008)
Recombinant dual-specificity tyrosine phosphorylation-regulated kinase 1	DYRK1	S329	Inactivation, nuclear exclusion	Woods et al. (2001)
Nemo-like kinase	NLK	S329	Inactivation, nuclear exclusion	Kim et al. (2010)
Casein kinase 1	CK1	S325	Inactivation, nuclear exclusion	Rena et al. (2002)
Mitogen-activated protein kinase-activated protein kinase 5	MK5	S215	Activation	Chow et al. (2013)
Protein kinase R-like endoplasmic reticulum kinase	PERK	S298	Activation, nuclear localization	Zhang et al. (2013)

3.1. FOXO1 in Bacteria-Epithelial Interactions. The epithelium acts as a physical barrier to prevent the entry of bacteria into the underlying connective tissues [35]. Dysbiotic bacteria-epithelial interactions disrupt the integrity of the periodontal tissues. Previous studies have demonstrated that *P. gingivalis* can induce increased expression and activity of FOXO1 in gingival epithelial cells [36, 37]. The upregulation and activation of FOXO1 lead to the production of AMPs by the epithelium and to the elevated levels of antioxidant genes (e.g., CAT, SOD2, and PRDX3), apoptotic genes (BCL-6, BID, and TRADD), toll-like receptors (TLR-2 and TLR-4), and integrins, which together contribute to the control of potentially pathogenic bacteria [36, 37]. Interestingly, regulation of apoptotic genes by FOXO1 depends on *P. gingivalis* exposure time. Short-time exposure increases the antiapoptotic gene (BCL-6), while long-term exposure reduces proapoptotic genes (BID and TRADD) [36, 37]. In addition, upregulation of zinc-finger E-box-binding homeobox 2 (ZEB2) by *P. gingivalis* in gingival epithelial cells is also mediated through the pathway involving FOXO1. The homeostatic commensal *Streptococcus gordonii* can suppress FOXO1 induction and antagonize ZEB2 induction by *P. gingivalis* via activating the TGF-beta-activated kinase 1-Nemo-like kinase (TAK1-NLK) pathway [38] (Figure 2). Collectively, these results suggest that FOXO1 comprises a component of host epithelial response to periodontal bacteria. However, activation mechanisms and biological impact on the epithelium remain to be understood in future studies.

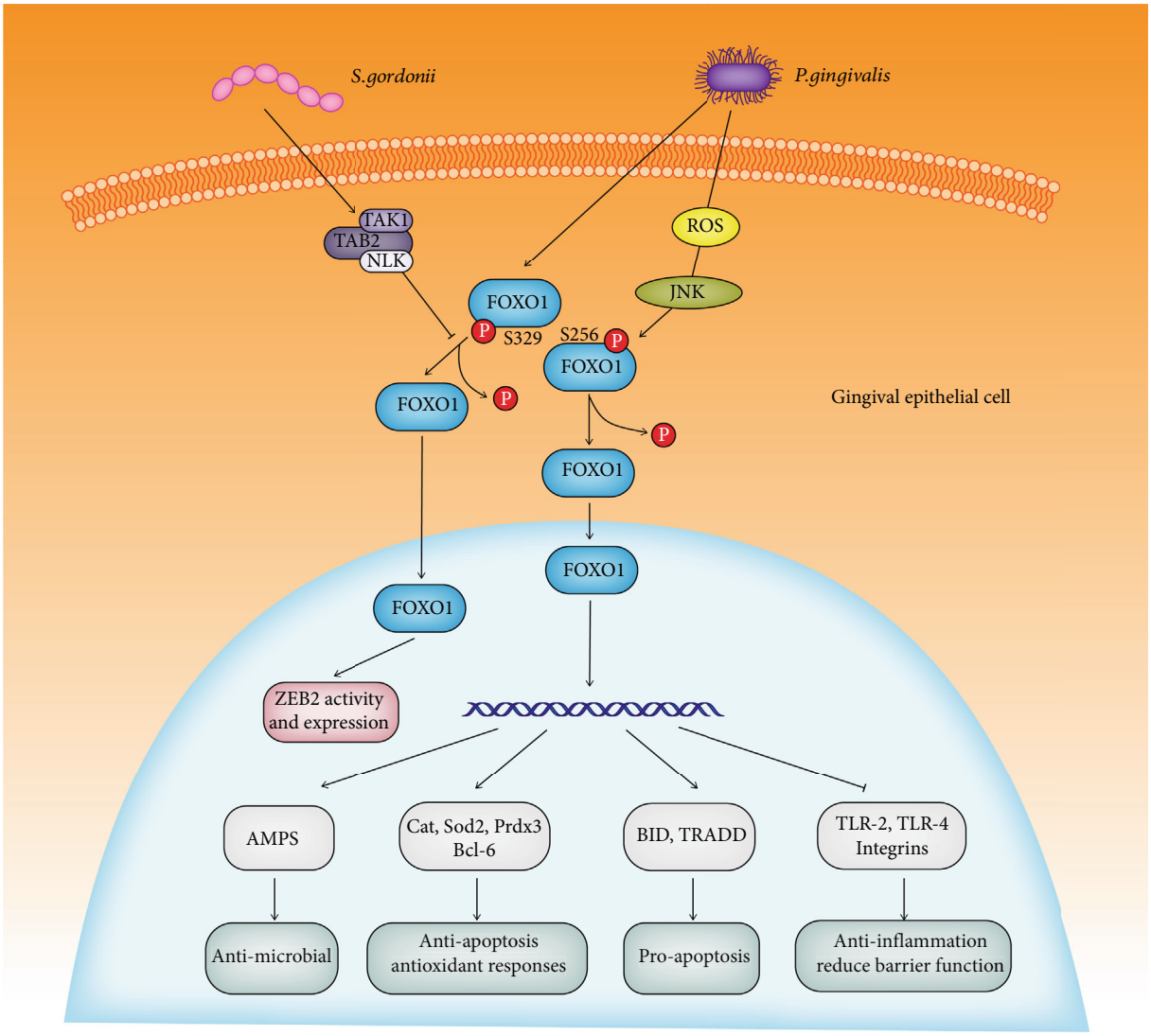

Figure 2: Regulation of FOXO1 activity by periodontal microbiota in gingival epithelial cells. FOXO1 (S256) can be dephosphorylated by *P. gingivalis* via ROS-JNK, promoting the nuclear localization of FOXO1. In the nucleus, FOXO1 mediates gene expression related to antimicrobial, antiapoptosis (Bcl-6), antioxidant response (Cat, Sod2, and Prdx3), proapoptosis (BID, TRADD), anti-inflammation (TLR-2 and TLR4), and epithelial barrier function. FOXO1 (S329) can also be dephosphorylated during *P. gingivalis* infection, resulting in the activation of ZEB2, and this process can be inhibited by the negative role of TAK1-NLK pathway, which is activated by *S. gordonii*.

Additionally, the role of FOXO1 in organizing the epithelium response to the subgingival plaque *in vivo* is still missing.

3.2. FOXO1 in Immune-Inflammatory Responses. In the periodontally healthy state, host-bacteria interactions are balanced, and when homeostasis is disrupted, the innate and adaptive immune systems work in concert in recognition and disposal of the periodontal bacteria. The role of FOXO1 in the immune system, especially dendritic cells, T cells, and B cells, has been comprehensively reviewed [39]. Here, we mainly focus on the potential relevance of FOXO1 for periodontal homeostasis and disease.

3.2.1. FOXO1 in Innate Immune Responses. The innate immune response to the invading bacteria is mediated mainly by phagocytes, NK cells, and DCs. After contact with phagocytes, initially neutrophils and later macrophages, bacteria generally are rapidly ingested and killed inside the cell. Some organisms are resistant to degradation within phagocytes, which cause the activation of NK cells. NK cells can also be activated by DCs [8].

In physiological conditions, neutrophils, which constitute ≥95% of total leukocytes in the gingival crevice, form a defense "wall" which protects the underlying tissues from periodontal bacteria [40, 41], and patients with neutrophil defects are more susceptible to periodontal disease [42]. Previous studies have provided initial evidence that FOXO1 may favor the survival and recruitment of neutrophils [43, 44]. For instance, Yang et al. found that FOXO1 is capable of forming a complex with myeloid cell leukemia-1 (MCL-1) and coordinate neutrophil survival [44]. In agreement, FOXO1 is also needed to mobilize neutrophils from the bone

marrow to the vasculature and to recruit neutrophils to sites of bacterial infection [43]. Moreover, the potential role of FOXO1 in macrophage polarization has also been studied. Highly expressed FOXO1 was found in M2 macrophages, and M2-like macrophages show FOXO1 enrichment on the IL-10 promoter following lipopolysaccharide (LPS) treatment [45]. Further, both Sirtuin 3 (SIRT3) and TGF-β-mediated macrophage M2-like polarization can occur via FOXO1-dependent pathways [46, 47]. Taken together, it is possible that FOXO1 promotes macrophage polarization towards the M2-like phenotype, thus suppressing inflammation and facilitating wound repair. Interestingly, in high-glucose conditions, macrophages exhibit an inflammatory phenotype, which is possibly due to reduced binding of FOXO1 to the promoter region of IL-10 [45]. Conversely, it has also been reported that FOXO1 activation can abolish M2 macrophage polarization and induce proinflammatory cytokine IL-1β expression [48]. In particular, FOXO1 is capable of binding to the IL-1β promoter and enhancing IL-1β promoter activity [48]. RNAi-mediated FOXO1 knockdown results in abrogation of the FOXO1-mediated induction of IL-1β promoter activity in LPS-stimulated macrophages [48]. IL-1β is a multifunctional cytokine that not only directly affects the regulation of various genes that are characteristically expressed during inflammation but also indirectly affects the stimulation of various cells to produce connective tissue catabolic and bone-resorptive mediators [14, 49]. This cytokine is also involved in osteoclast formation and bone resorption by inducing RANKL [50]. Chen et al. have also reported that FOXO1 is indispensable for protease-activated receptor 2 (PAR2) promotion of M1 macrophage polarization [51]. Thus, under these experimental conditions, FOXO1 is indispensable for promoting M1 macrophage polarization. These apparently contradicting functions may be reconciled if the role of FOXO1 in macrophages depends on the conditions. More importantly, the role of FOXO1 in macrophages needs to be directly tested *in vivo*. Recently, FOXO1 has been defined as a negative regulator of NK cell maturation and effector function [52]. The LPS of *P. gingivalis* can promote the proliferation and activation of NK cells *in vivo*. In turn, the NK cells produce IFN-γ, which can activate macrophages and promote killing of phagocytosed bacteria [53]. Thus, it is tempting to speculate that FOXO1 may attenuate NK cell-mediated periodontal bacterial killing. However, the role of NK cells is complex and additional studies are still necessary. Additionally, DCs can induce a protective response through induction of Th2 lymphocytes [54]. However, they may also potentially enhance periodontal bone loss through upregulation of Th1 or Th17 responses [55]. The linkage between FOXO1 and DCs has been well studied and systematically reviewed by Graves et al. [56]. In brief, FOXO1 is activated in DCs and it is crucial for DC homing to lymph nodes, binding to lymphocytes and formation of an immune synapse which activates lymphocytes [57, 58]. FOXO1 nuclear localization and activity are induced by the MAPK pathway and inhibited by PI3K/AKT [56] (Figure 3(a)). The role of FOXO1 in periodontal homeostasis and dysbiosis via DCs is condition dependent. With lineage-specific FOXO1 deletion mice, Graves et al. have demonstrated that decreased FOXO1 reduces the recruitment of DCs to the gingiva and impairs the function of DCs both in normal and in aging mice. Specifically, FOXO1 deletion reduced migration of DCs to lymph nodes and decreased IL-12 production at mucosal surfaces [58]. Moreover, FOXO1 induces transcriptional activity and stimulates expression of the adhesion molecule intercellular cell adhesion molecule-1 (ICAM-1), integrins αv and β3, C-C chemokine receptor 7 (CCR7), and matrix metalloproteinase-2 (MMP-2), all of which are needed for the activity of DCs [57]. Interestingly, when challenged by oral infection, FOXO1 deletion reduced the adaptive immune response of DCs in normal mice. Aging is associated with decreased FOXO1; however, increased adaptive immune response was observed in aged mice compared with young mice, and the increase was reversed by FOXO1 deletion in DCs.

3.2.2. FOXO1 in Adaptive Immune Responses. Adaptive immunity is thought to have evolved to provide a focused and intense defense against infections that overwhelm innate immune responses [59]. Usually, the failure of the innate immune response to control periodontal infection results in the recruitment of T cells and B cells to the periodontium. The presentation of bacteria or bacterial antigen captured by specialized antigen-presenting cells (APCs), such as macrophages and DCs, activates T cells and B cells. As specialized APCs, activated DCs produce various cytokines, including IL-1, IL-6, IL-10, IL-12, IL-23, IL-27, and TNF-α, which affect the activation and biological activity of other innate and adaptive immune cells [60] (Figure 3(a)). Thus, the positive regulation of FOXO1 in DCs as mentioned above may also influence this process.

Several studies have also revealed that FOXO1 participates in the differentiation and metabolism of T cells. FOXO1-deficient T cells stimulated by transforming growth factor-β (TGF-β) *in vitro* show compromised Treg cell differentiation. *In vivo*, T cell-specific FOXO1-deficient mice showed decreased frequency and number of thymic Tregs among $CD4^+$ T cells [61]. One possible mechanism of FOXO1 guiding the differentiation of $CD4^+$ T cells relates to the PI3K-mTORC2-AKT signaling pathway [61, 62] (Figure 3(b)). $CD4^+$ T cells can activate phagocytes through the action of the CD40 ligand (CD40L) and IFN-γ, resulting in bacterial elimination and cytokine production. A higher proportion of Tregs has been observed in peripheral blood samples and periodontal tissue samples from chronic periodontitis patients compared to those from healthy individuals [29, 63]. Inhibition of Treg function in the periodontal tissue of mice results in increased alveolar bone loss and inflammatory cell migration, associated with decreased anti-inflammatory cytokine production along with increased inflammatory cytokine (IFN-γ and IFN-α) and RANKL production [64]. In this regard, FOXO1-mediated T cell differentiation is considered as a protective response against advanced infection [65, 66]. Otherwise, excessive T cell-mediated recruitment and activation of phagocytes and cytokine production are capable of causing tissue injury, such as vascular changes associated with

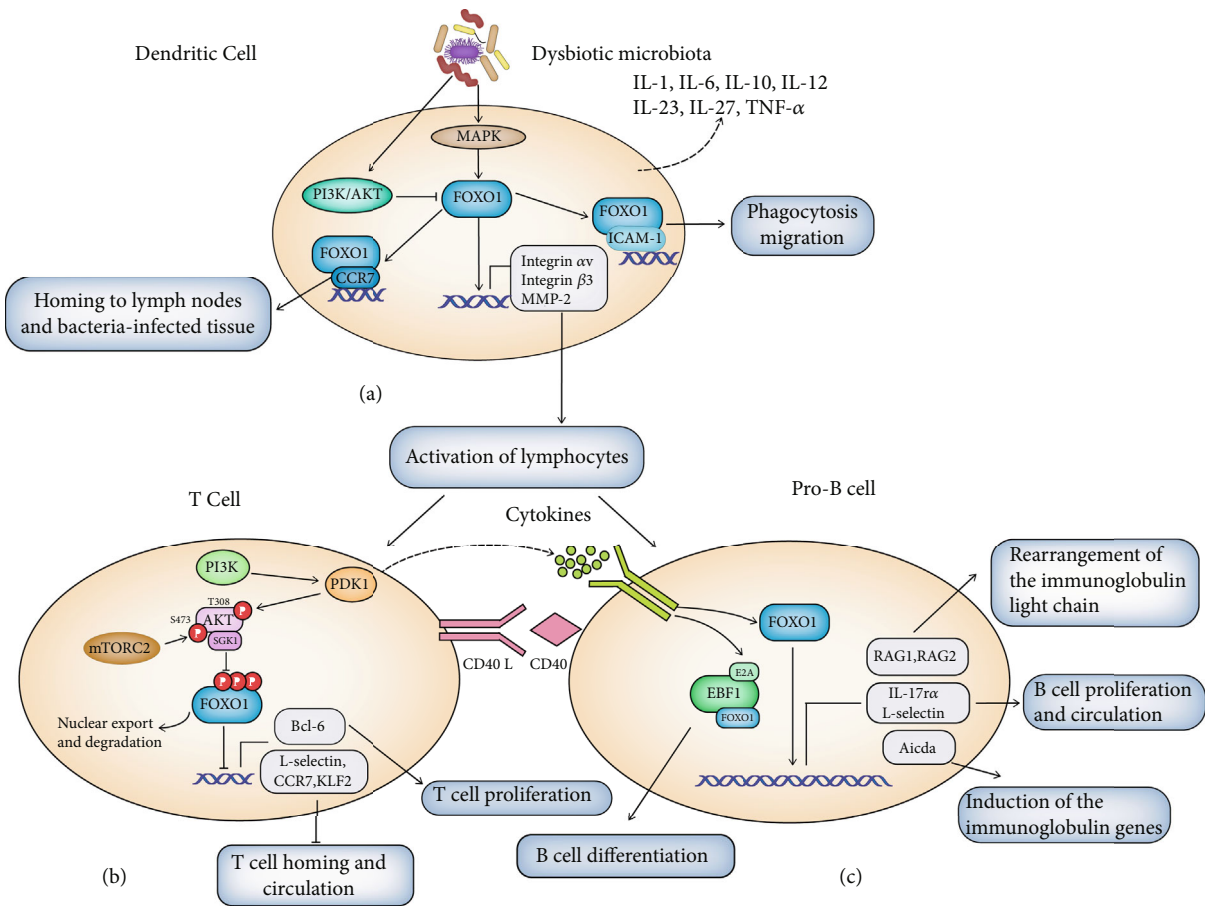

Figure 3: FOXO1 participates in function of DCs, T cells, and pro-B cells. (a) Dysbiotic microbiota stimulation initiates a signaling cascade to regulate the activity of FOXO1 via the MAPK and PI3K/AKT pathways. Activated FOXO1 can upregulate the transcription of CCR7, ICAM-1, integrin αv, integrin β3, and MMP-2, thus homing DCs to lymph nodes and bacteria-infected tissue, promoting phagocytosis migration and activation of lymphocytes. (b) FOXO1 associates with T cell proliferation, T cell homing, and circulation, which depend on counteracting the PI3K-, mTORC2-, and AKT-dependent negative regulation of FOXO1. (c) In pro-B cell, FOXO1 can be activated by various cytokines and participates in rearrangement of the immunoglobulin light chain, B cell proliferation and circulation, and B cell differentiation by promoting expression of key target genes, such as RAG1, RAG2, IL-17rα, L-selectin, Aicda, and EBF1.

inflammation, bone resorption, and the infiltration of neutrophils into affected tissues [62].

Another role of FOXO1 involves T cell-secreted cytokines and the interaction of CD40L on $CD4^+$ T cells with CD40 on the B cell surface, which results in the activation of B cells (Figure 3(c)). FOXO1 has been identified by Dengler et al. as the master transcriptional regulator that orchestrates the differentiation, activation, and proliferation of B cells [67]. Specifically, FOXO1 is upregulated during the early pro-B cell stage, and a decrease in FOXO1 protein levels impairs several stages of B cell development through regulation of key target genes, such as IL-17 receptor alpha (IL-17rα), recombination-activating gene 1 (RAG1) and 2 (RAG2), L-selectin, Aicda, and early B cell factor (EBF1) [68, 69]. In activated B cells, FOXO proteins exert their effects via the upregulation of both proapoptotic genes (e.g., BIM and BCL-6) and antiproliferative genes (e.g., P21 and P27) [70, 71]. Optimal B cell proliferation requires PI3K-dependent inactivation of FOXO transcription factors [72]. Thus, FOXO1 may play key roles in regulating T and B cells in a highly cell- and context-specific manner.

Collectively, progress in the field of FOXO1 in immune regulation has revealed its versatile and condition-dependent functions for periodontal homeostasis and disease. Physiologically, FOXO1 seems to be critical for the recruitment of neutrophils, polarization of macrophages, homing and function of DCs, and differentiation of T cells and B cells. It may function to respond to environment changes and work to counteract the potential damage caused by high glucose, bacterial infection, and aging through regulating immune responses. It will be of interest to study the lineage-specific FOXO1 knockout model further to identify the role of FOXO1 in polarization of macrophages. The linkage between FOXO1 and DCs has been well studied by Graves et al. in periodontal tissues. However, there is still no direct evidence demonstrating the activation and function of FOXO1 in other immune cells for periodontal homeostasis and disease. In particular, further exploration of FOXO1 in immune responses under pathological conditions such as periodontitis and diabetes mellitus will be important to establish the full involvement of FOXO1.

3.3. FOXO1 in Alveolar Bone Remodeling. The alveolar bone is part of the maxilla and mandible that forms and supports the tooth socket. It develops around each tooth follicle during odontogenesis. As the permanent tooth root forms and the surrounding tissues develop and mature, alveolar bone merges with the basal bone [73]. In physiological conditions, the alveolar bone is in the process of continuous reconstruction. Bone deposition by osteoblasts and bone resorption by osteoclasts maintain a dynamic balance during tissue remodeling and renewal. When the concentration of inflammatory mediators in the gingival tissues reaches a threshold, the pathways that lead to bone resorption will be overactivated and bone loss will occur [8, 73]. Interestingly, recent studies have also demonstrated that osteoblast lineage cells are critical for periodontal bone resorption by increasing the number of osteoclasts as well as osteoclast activity [74].

3.3.1. FOXO1 in Bone Deposition and Bone Coupling by Osteoblast Lineage Cells. FOXO1 is highly expressed in osteoblasts under physiological conditions [75]. Conditional deletion of FOXO1 in osteoblasts can cause a decrease in osteoblast numbers, bone formation rate, bone volume, and bone mineral density in the spine and femur of mice. Notably, the influence of FOXO1 as a regulator of bone mass is specific among all FOXO proteins [75–77] as FOXO1 is thought to positively regulate new bone formation in osteoblasts by favoring resistance to excessive levels of ROS and counteracting deleterious consequences of oxidative stress on the cells [75, 77, 78]. Specifically, deletion of FOXO1 in mouse osteoblasts results in decreased expression and activity of superoxide dismutase 2 (SOD2), accompanied by elevated levels of ROS and lipid peroxidation end products [77]. Moreover, supplying the antioxidant N-acetyl L-cysteine (NAC), which can normalize redox levels, leads to the phenotypic bone abnormalities of FOXO1 osteoblast knockout mice as mentioned above [77]. These effects of FOXO1 relate to its role in regulating the activity of signal transduction pathways activated by ROS, p53, and p66shc [77]. FOXO1 can reduce the activity of P53 by inhibiting the expression of P19ARF and P16, thus mediating ROS-induced antiproliferative actions [79]. Similarly, FOXO1 can also inhibit the activity of p66shc and influence proapoptotic action of ROS [80]. Deletion of FOXO1 in osteoblasts also compromises amino acid import and protein synthesis, thus resulting in decreased levels of glutathione (GSH) and in a subsequent increase in ROS. This is associated with FOXO1-ATF4 interaction [77]. Interestingly, under conditions of a strong host response induced by *P. gingivalis*, FOXO1 has been reported as a proapoptotic factor, which was sustained and highly activated by the acquired immune response, thus inducing increased apoptosis of osteoblast and reduced bone coupling [81].

3.3.2. FOXO1 in Bone Resorption by Osteoclasts. Intracellular H_2O_2 accumulation is critical for the differentiation and survival of osteoclasts. As a sensor, mediator, and regulator of redox signaling, FOXO1 is elevated in conditions with high levels of bone resorption and has the ability to regulate the formation and activation of osteoclasts [27, 81, 82]. Bartell et al. found that long-term combined deletion of FOXO1, FOXO3, and FOXO4 decreases physical bone mass by increasing osteoclast numbers and activity [83]. Furthermore, FOXO1 suppressed bone resorption by attenuating H_2O_2 accumulation [83]. Consistent with this study, Tan et al. demonstrated that FOXO1 acts as a cell-autonomous inhibitor of osteoclast differentiation and activity, which is partially mediated by MYC suppression [84]. However, the regulation of osteoclast formation and activity is a complex process, which is affected by multiple factors, and moreover, the same factor may play different roles in this process. Therefore, studies on the role of FOXO1 in osteoclasts generate disparate results with different approaches. For instance, Wang et al. found that FOXO1 is a direct player in osteoclast formation and activity by mediating the action of RANKL on NFATc1 and several downstream effectors, including dendritic cell-specific transmembrane protein, ATP6vod2, cathepsin K, and integrin αv. Lineage-specific deletion of FOXO1 in osteoclast precursors (LyzM. $Cre^{+}FOXO1^{L/L}$) leads to reduced RANKL-induced osteoclast formation and osteoclast activity [85].

3.3.3. FOXO1 in Modulating Osteoblast Differentiation. Osteoblast differentiation is controlled by various external signals that induce a cascade leading to terminal differentiation of osteoblasts from mesenchymal cells and osteoblastic precursors [86]. Accumulating evidence indicates the involvement of FOXO1 in osteoblast differentiation [75, 87, 88]. In physiological conditions, FOXO1 plays an important role in promoting osteoblast differentiation, maintaining normal erythropoiesis and hematopoietic stem cell quiescence and survival. Siqueira et al. studied the role of FOXO1 in modulating osteoblast differentiation by a system in which preosteoblastic cells undergo terminal differentiation [87]. They reported upregulation of FOXO1 mRNA levels and DNA binding activity in normal cells during formation of mineralizing nodules. Interestingly, overexpression of FOXO1 reduced MC3T3-E1 cell number and the number of proliferating cell nuclear antigen-positive cells. Teixeira et al. found similar results with FOXO1 expression and activity increasing in mouse bone marrow mesenchymal stem cells (BMSCs) [88]. FOXO1 can also affect mesenchymal cell differentiation into osteoblasts by directly interacting with the promoter of RUNX2 and increasing its expression, further confirming the function of FOXO1 in osteoblast differentiation [88]. In addition, conditional deletion of FOXO1 in developing mice results in excessive levels of ROS in the bone and increased osteoblast apoptosis and reduced number of osteoblasts [75].

Conversely, a series of findings reported by another team suggest that FOXO1 activation can eventually aggravate the effects of oxidative stress on the bone. Almeida et al. reported that oxidative stress promotes the association of FOXO1 with β-catenin, thus suppressing Wnt-/T cell factor-mediated transcription and osteoblast differentiation [89]. Later, Iyer et al. confirmed this finding in vivo with mice lacking FOXO1 in bipotential progenitors of osteoblast and adipocytes, which suggest that FOXO1 could attenuate Wnt signaling, thereby decreasing the number of matrix-synthesizing osteoblasts and amount of bone mass [90]. A possible explanation

for these contradictory results is that FOXO1 is an early molecular regulator in promoting differentiation of mesenchymal cells and preosteoblastic cells into osteoblasts. Age-related increased oxidative stress may stimulate FOXO1, therefore determining the role of FOXO1 in osteoblast differentiation.

Given the pivotal role FOXO1 can play in new bone formation and bone coupling by osteoblast lineage cells, resorption of the mineral matrix by osteoclasts, and differentiation and proliferation of precursor cells, it is potentially a very relevant player in alveolar bone remodeling. Besides, ROS levels fluctuate significantly in different periodontal microenvironments; thus, the critical role for FOXO1 in bone remodeling also indicates that FOXO1 may be involved in alveolar bone remodeling by fighting against oxidative stress. However, significant gaps exist in demonstrating the expression and activity of FOXO1 in alveolar bone whether in physiological or pathological conditions. Further, there is a need to understand the precise mechanism of FOXO1 in the alveolar bone remodeling process.

3.4. FOXO1 as a Key Player in Periodontal Wound Healing. The keratinized epithelium of the gingival and sulcular epithelial tissues acts as a barrier against invasion of periodontal bacteria and their products and provides protection for the underlying periodontal connective tissue [12]. There is also an impermeable seal of junctional epithelium and connective tissue between the external environment and the internal parts of the body [12, 91]. When periodontal tissues are damaged, a sequentially phased wound healing response is initiated. This process usually consists of four steps: hemostasis, inflammation, proliferation and granulation, and finally maturation of renewal tissue for remodeling. Periodontal tissue wound healing is similar to the healing process of skin epithelium, which is a complex scenario that involves the tightly regulated coordination of resident cells in epithelial and connective tissues, as well as cytokines, growth factors, and extracellular matrices [91]. Furthermore, continuously elevated proinflammatory mediators may result in excessive formation of disorganized connective tissue matrices. In addition, systemic host factors such as diabetes mellitus influence on wound healing, and poorly controlled diabetics often have disordered wound healing [92].

Within hours of injury or surgery, epithelial cells of the basal layer proliferate and migrate through the fibrin clot and breach [91]. The role of FOXO transcription factors in epithelial wound healing has been reviewed [93, 94]. In brief, FOXO1 differentially regulates both normal and diabetic wound healing. In normal healing, FOXO1 promotes epithelial cell proliferation and migration by upregulating the expression of transforming growth factor-β (TGF-β) and its downstream targets such as integrins (integrin α3 and integrin β6) and matrix metalloproteinases (MMP-3 and MMP-9), as well as reducing oxidative stress [93, 95]. Recent evidence suggests that high levels of ROS and subsequent oxidative stress are key contributors to the development of periodontal diseases [96]. During the inflammatory stage of wound healing, neutrophils produce large amounts of ROS [97], which result in oxidative stress and subsequently increase apoptosis in the deepest area of sulcular pockets [98], causing further induction of proinflammatory cytokines and DNA damage [96]. It has been shown that elevated intracellular ROS increases nuclear localization and thus activity of FOXO1 through c-Jun-N-terminal kinase (JNK) signaling in gingival epithelial cells, which can induce the expression of genes that counter oxidative stress (CAT, SOD2, and PRDX3) and apoptosis (BCL-6) [37]. Thus, we speculate that FOXO1 is a positive regulator of periodontal wound healing in normal conditions. Another mechanism by which FOXO1 may be involved in gingival epithelial wound healing is via promoting angiogenesis. Deletion of FOXO1 in keratinocytes causes reduced endothelial cell proliferation and impaired angiogenesis. These effects correlate with the decreased expression of vascular endothelial growth factor A (VEGFA) [99]. In addition, decreased type I collagen density accompanied by reduced collagen fiber organization was found at the wound site in FOXO1$^{+/-}$ mice [100]. Collagen, especially type I collagen, is the major structural protein for gingival connective tissue. It is also a key component in wound healing by providing a biologic scaffold for cellular activities such as cell attachment, migration, proliferation, and synthesis of a number of proteins. In contrast to the positive function of FOXO1 in wound healing, another study demonstrated the inhibitory role of FOXO1 in normal wound healing [101]. This study reported that acute knockdown of FOXO1 could promote early stage epithelial wound healing by increasing the expression of proteins critical for reepithelialization, including fibroblast growth factor 2 (FGF2), adipoq, Notch 1, and Myosin X (MYO10) [101]. The disparate results of FOXO1 in normal wound healing may be caused by different methods for FOXO1 knockdown as, for example, keratinocyte-specific FOXO1 deletion is more suitable for periodontal wound healing. In addition, it is generally accepted that poorly controlled diabetes has an adverse effect on periodontal wound healing, which is also partially mediated by FOXO1. In high-glucose conditions, FOXO1 is also activated which causes delayed wound healing by increasing expression of C-C chemokine ligand (CCL20) [102].

Altogether, the evidence suggests that the potential role of FOXO1 as a key cell regulator during periodontal wound healing may depend upon the specific microenvironment. In normal conditions, FOXO1 may function as a positive regulator via the following possible mechanisms: (1) improving cellular antioxidant capacity and suppression of apoptotic cell death, thus positively regulating proliferation and migration of epithelial cells; (2) promoting angiogenesis; and (3) inducing collagen synthesis. However, in high-glucose conditions, there is an opposite effect of FOXO1 through induction of inflammatory gene expression.

3.5. The Role of FOXO1 in Periodontal Tissue Homeostasis. It is clear from the studies *in vitro* and *in vivo*, FOXO1 is sensitive to the environmental changes (oxidative stress and glucose level), which are closely related to periodontal homeostasis maintenance. More interestingly, a recent study found that FOXO1 exerted antioxidative effort on protecting human periodontal ligament stem cells (hPDLSCs) from cellular oxidative damage and promoting osteogenic

differentiation capacity of hPDLSCs in the inflammatory microenvironment [103]. Therefore, in the complex scenario of host-microbe interactions, immune response, bone remodeling, and wound healing associated with periodontal disease, FOXO1 might contribute to periodontal tissue homeostasis at multiple levels in a context- and condition-dependent manner. Generally, FOXO1 can be involved in the following processes: (1) protecting gingival epithelial cells from oxidative damage and antiapoptosis in response to periodontal bacterial challenge; (2) inducing recruitment of neutrophils, homing and function of DCs, and differentiation of T cells and B cells in physiological conditions; (3) promoting bone deposition by osteoblast and osteoblast differentiation; and (4) accelerating epithelial wound healing in normal conditions. Therefore, we hypothesized that in periodontitis, periodontal tissue damage caused by excess oxidative stress response, inflammatory immune response, and impaired osteogenesis can be accomplished by a decrease in the activity of FOXO1. However, how to use the FOXO1 transcription factor in potential therapeutics still needs further exploration.

4. Conclusions

Periodontitis is a chronic inflammatory disease that is characterized by destruction of the tooth-supporting structures, such as gingivae, periodontal ligaments, and the alveolar bone. FOXO1 is implicated in bacteria-epithelial interactions, immune status, bone remodeling, and wound healing, all of which have direct relevance for periodontal homeostasis and dysbiosis. These include improving cellular antioxidant capacity and suppression of apoptotic cell death; recruitment of neutrophils to sites of bacterial infection and DCs to the oral mucosal epithelium; regulation of macrophage polarization and NK cell maturation; the differentiation of adaptive immune cells, such as Tregs and B cells; modulation of bone deposition by osteoblasts and bone resorption by osteoclasts as well as osteoblast differentiation; and regulation of wound healing. Therefore, FOXO1 may mainly function as a homeostatic regulator which coordinates responses to environmental signals that disturb the periodontal homeostasis. However, direct evidence for the mechanism of FOXO1 action in periodontal tissues under both physiological and pathological conditions requires further study.

Data Availability

Data are available on request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

LR and JY drafted the manuscript and prepared the figures and tables; JW and XZ drafted parts of the manuscript and critically revised the manuscript; CL reviewed, edited, and approved the final version of the manuscript; all authors reviewed, edited, and approved the final version of the manuscript. Liang Ren and Jing Yang contributed equally to this article.

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