Notch/NICD/RBP-J signaling axis regulates M1 polarization of macrophages mediated by advanced glycation end products

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Abstract
Advanced glycation end products (AGEs) aggregation and macrophages polarization are identified as the main factors contributing to bone diseases caused by aging or diabetes, such as senile or diabetic osteoporosis. Here, we aimed to elucidate the involvement and potential mechanism of AGEs in macrophages polarization and osteoclastogenesis. Firstly, AGEs-treated RAW264.7 macrophages were observed to up-regulate the release of nitric oxide (NO), the expression of M1-associated genes and the surface antigen marker CD86. The detection of osteoclast-related markers and TRAP staining revealed that the osteoclastogenic ability of M1 macrophages was markedly enhanced by AGEs. Further, AGEs were found to effectively activate the transduction of Notch signaling pathway and promote the nuclear translocation of NICD1. In addition, with the signals transduction of Notch pathway blocked by γ-secretase inhibitor DAPT and siRNA targeting silencing RBP-J, AGEs-induced M1 polarization was significantly mitigated. Collectively, we defined a critical role for AGEs in inducing M1 polarization and osteoclastogenesis of macrophages, and further identified Notch/NICD/RBP-J signaling axis as an essential mechanism regulating AGEs-mediated M1 polarization.

Keywords Advanced glycation end products (AGEs) · Macrophage polarization · Notch signaling · Osteoclastogenesis

Introduction
Advanced glycation end products (AGEs) are a variety of compounds formed by the non-enzymatic reaction of reducing sugars (such as glucose) and some metabolites (such as methyl adipaldehyde) with protein amino groups. AGEs are continuously accumulated in the body through exogenous ingestion or endogenous generation[1, 2]. Excessive accumulation of AGEs accelerate the aging of human body, leading to the occurrence of certain chronic degenerative diseases such as diabetes, senile osteoporosis, Alzheimer’s disease, etc.[3, 4]. Notably, the interaction between AGEs and receptors expressed by antigen presenting cells (APCs) is considered to be one of the key immunomodulation mechanisms mediating the above diseases[5].

Macrophages, one of the professional APCs, are the main members of the innate immune system. Macrophages are sufficiently dynamic and plastic to integrate a variety of microenvironmental signals and are functionally related to various diseases[6]. It is well known that macrophages undergo either classically activation (M1) or alternatively activation (M2) in response to various microenvironmental cues[7]. M1 macrophages are characterized by the pro-inflammatory effects and play an essential role in host resistance to infection by secreting inflammatory factors, including induced nitric oxide synthase (iNOS), interleukin-6 (IL-6), and interleukin-12b (IL-12b). In contrast, M2 macrophages mainly secrete anti-inflammatory cytokines such as interleukin-10 (IL-10) and Arginase-1 (Arg-1) to inhibit inflammation and promote wound healing [8]. Interestingly, macrophages also control the fate of osteoclasts and influence the homeostasis of bone metabolism[9]. It has been long recognized that macrophages polarization is closely associated osteoclastogenesis[10, 11].

Emerging evidences suggest that AGEs are involved in the regulation of macrophages polarization. It has been
reported that AGEs promote M1 polarization of macrophages through RAGE or energy metabolism pathway [12, 13]. However, according to another research, AGEs were reported to weaken the activation of inflammmasomes and inhibit the M1 polarization of macrophages induced by lipopolysaccharide [14]. Therefore, the specific effects of AGEs on macrophages polarization are still inconclusive, and the latent mechanisms also remain obscure. In addition, AGEs also affect the resorption activities of osteoclasts and impair the bone metabolic balance[15]. Hence it is of great significance to uncover the potential association between macrophages polarization and osteoclasts metabolism under the action of AGEs.

Notch signaling pathway, characterized by a high degree of conservatism, widely participates in the development and differentiation of organs, tissues, and cells, especially in regulating the function of immune system cells such as monocytes, macrophages, and lymphocytes [16]. The pathway consists of a series of known receptors (Notch1, Notch2, Notch3), ligands (Jagged1, Jagged2, DLL1, DLL3, DLL4), related enzymes, and intracellular effector molecules (Hes/Hey family) [17, 18]. Notch signaling is also considered to be a key regulator of the biological function of macrophages [19]. Studies have demonstrated that Notch signaling on macrophages can regulate the differentiation of macrophages into M1 and activate macrophages to release more cytokines and chemokines for promoting inflammatory responses [20]. Although Notch signaling has been confirmed to regulate the macrophages polarization, its role in AGEs-mediated macrophages polarization remains unreported.

Herein, we aimed to clarify the involvement of AGEs in the polarization and osteoclastogenesis of macrophages and sought to identify the molecular mechanism of Notch signaling pathway in regulating macrophage plasticity.

Materials and methods

Preparation of AGEs

AGEs were synthesized with support of Beijing Bios Bio-technology Co., Ltd. Briefly, the mixed solution of BSA (bovine serum albumin) and glucose was incubated at 37°C under sterile conditions for 30 days, during which BSA was gradually glycated in a high glucose environment. The concentration of AGEs was estimated by a BCA protein assay kit (Beyotime, China). As a control, non-glycated BSA was prepared by the same procedure but without glucose.

Cells culture

Murine macrophage lines RAW264.7 were purchased from American Type Culture Collection (ATCC, USA) and maintained in α-MEM (Sigma, USA) supplemented with 10% fetal bovine serum (FBS, Lonsera, Uruguay) and 1% antibiotics (penicillin 100U/mL, streptomycin 100 µg/mL, ) providing atmosphere of 37°C and 5% CO2. In order to research macrophages polarization, cells were treated with different concentrations of AGEs (Bioss, China), 1 µg/mL LPS (Solarbio, China) and 20mg/mL IL-4 (Sino Biological, China). To induce macrophages to differentiate into osteoclasts, α-MEM containing 50ng/mL RANKL (Novoprotein, China) and 30ng/mL M-CSF (Novoprotein, China) was used to culture cells for 6 days. Three repeat holes were set for each sample.

Cells viability and toxicity test

Cell Counting Analysis Kit (CCK-8, Beyotime, China) for cell viability detection was performed according to the manufacturer’s instructions. Briefly, RAW264.7 cells were seeded in a 96-well plate at a density of 5 × 10^3/well. After 24 h, the medium was replaced with fresh α-MEM medium containing AGEs at concentrations of 100, 200, 400 mg/L for 12, 24, 36, and 48 h. Then, after the supernatant was discarded and cells were washed with PBS twice, 10µL CCK-8 solution was added to each well and cultured at 37 °C for 3 h. The absorbance was measured by enzyme labeling instrument (PerkinElmer, USA) at 450 nm wavelength.

An Annexin V-FITC apoptosis assay kit (Beyotime, China) was used to estimate the apoptosis rate of RAW264.7 cells. Briefly, cells in logarithmic growth phase were inoculated in 6-well plates and cultured for 24 h. Then the medium was replaced with fresh α-MEM medium containing AGEs at concentrations of 100, 200, 400 mg/L for 24 h. After washed with PBS twice, cells were stained with Annexin V-FITC and propidium iodide (PI) in binding buffer at 4°C. Samples were detected with flow cytometer (BD Influx, USA) and the results were statistically analyzed by FlowJo.v10.6.2.

Griess assay

First, a standard curve was drawn using NO standards according to the instructions of the Griess kit (Beyotime, China). After the cells were treated with 100 mg/L AGEs for 24 h, the cell supernatant of each group was extracted and added to a 96-well plate at 50µL/well. According to the instructions, 50µL of Griess Reagent I and 50µL of Griess Reagent II were added to each well, respectively. Then, the absorbance was measured at 540 nm wavelength with a
microplate reader and the NO concentration in the supernatant was calculated using the standard curve.

**Quantitative real-time PCR (qRT-PCR)**

For gene expression analysis, cells were washed twice by PBS and lysed in RNAiso plus (Takara, Japan). Total RNA was extracted using the TRizol method according to the manufacturer’s instructions and the RNA concentration was measured using NANO Drop2000 UV spectrophotometer (Thermo Scientific, USA). Then 2 µg of total RNA was reverse transcribed into cDNA using a 20µL system reverse transcription kit (Promega, USA). Quantitative real-time PCR (qRT-PCR) was then performed with CFX Connect real-time PCR detection system (Bio-Rad, USA) using qPCR Master Mix kit (Promega, USA). The relative expression of target genes was calculated and analyzed with the 2-ΔΔCT method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control and all reactions were performed in triplicate. The sequences of the primers (Sangon Biotech, China) are listed in Table 1.

**Western blot analysis**

Cells were lysed with RIPA buffer (Santa Cruz Biotechnology, USA) containing 1mM phenylmethylsulfonyl fluoride (Beyotime, China) after washed by cold PBS three times. The total protein concentration was quantified with an enhanced bichinchonic acid (BCA) protein analysis kit (Beyotime, China) and visualized using Chemi Doc Imaging System (Bio-Rad, USA). All images were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, China). Then the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Abbkine, USA) against monoclonal anti-iNOS, anti-Notch1, anti-cleaved Notch1, anti-RBP-J, anti-GAPDH and anti-Histone H3. Then the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Abbkine, USA) at room temperature for 1 h. After washing, the Western blot bands were treated by an ECL Plus chemiluminescence reagent kit (Beyotime, China) and visualized using ChemiDoc Imaging System (Bio-Rad, USA). All images were analyzed by ImageJ software (NIH, USA).

**Flow cytometry analysis for macrophages polarization**

Surface antigen expressions on RAW264.7 cells were determined by FCM. Briefly, cells were collected and suspended at 1 × 10^6 cells/mL with PBS containing 3% FBS. Then cell suspensions were incubated with PE anti-CD86 (BD Biosciences, USA) on a shaker at 4 °C for 30 min in the dark, during which time it was stirred every 5 min to avoid cell precipitation. After the incubation, unbound primary antibody was discarded and cells were washed twice with 500µL of PBS containing 3% FBS by centrifugation at 500 g for 5 min. Then the cell suspensions were filtered into flow cytometer tubes with a sieve and analyzed by flow cytometry (BD Influx, USA). The data were statistically analyzed by FlowJo.v10.6.2.

**Tartrate resistant acid phosphatase (TRAP) staining**

According to the protocols of the TRAP staining kit (Sigma, USA), cells were washed three times by PBS, then fixed

**Table 1**  Primers for Quantitative real-time PCR

| Gene(mouse) | Forward Primer (5’ to 3’) | Reverse Primer (5’ to 3’) |
|-------------|---------------------------|--------------------------|
| iNOS        | ACTCAGCCAAAGCCTT-GACCTAC | TCCAAATCTCT-GGCTATCC-GCTTCG |
| IL-6        | CTTCTTGAGCTGAT-GCTGGTAC  | AGGTCT-GTGGAGGTGT-GTATCTC |
| IL-12b      | CATTGAACGGCTTGG-GAGACAC  | GAGGGCGGCTG-GTTTGAATG |
| Arg-1       | GCCAACCTGT-GTCTTTCTTCGT  | GGTCTAGTCG-GCCAGCCAGG |
| IL-10       | TCTTTCACAA-CAAGGACCACG   | GCAACCCAGA-TCACCTTAAAAG |
| NFATc-1     | GGGTGGTCTTC-GAGTCCATAC   | GTGCTC-CTGTC-GCTTCTC |
| TRAF-6      | AGGAATCACTTGGCAG-GACACTTG | TGCTGTCG-TTACGCAG-GTCTC |
| RANK        | AGCCTCCGACGAGAAGCT-GAGTC | GTGCTGTCG-TAGCCACATGTG-GAG |
| TRAP        | CCACCTCAGATTTGCT-GCT     | ACATAC-CAGGAGATTT-GCG |
| CTSK        | CTGGAGGGACCACT-CAAGA     | CCTGCTGATT-TAGCTGCCTT |
| Jagged1     | AAAAACAGGAGAACA-CAACTCG  | CTGTTATTTT-GTCCAGTTCG |
| Notch1      | GTGCTGGAGATTT-TAGCGAC    | GTCTCTGACG-TACTGGTCATAC |
| RBP-J       | TCCACAGCCT-TACCTTTACCTAC | TTTGACTTATG-GAGGGCTAGT |
| Hey1        | CATGGACTATCG-GAGTGGGGTCTC | GGGATGG-CTACGTGGAG |
| GAPDH       | GGTGTGCTTCTTGT-GACTTC    | TGCT-CAGGTTTCT-TACTCC |
with 4% paraformaldehyde at room temperature for 20 min and washed again with PBS for three times. Then the staining solution mixture prepared according to the instructions was used to incubate cells for 60 min in the dark, after which the cells were rinsed three times with deionized water to avoid nonspecific staining. Then, mature osteoclasts, which manifested as positively stained cells with more than three nuclei, were counted under a light microscope (Leica DMI4000B, Germany).

**Treatment with DAPT**

DAPT (Abmole, USA), as a γ-secretase inhibitor, is usually used as an inhibitor of the Notch signaling pathway. While treating the cells with different stimuli, 5µmol/mL DAPT was added to the cell culture medium to inhibit the transmission of Notch signal, and then the polarization changes of the cells were detected.

**Gene interference with RBP-J siRNA**

Small interfering RNA (siRNA) targeting RBP-J (sense: 5’-GCCGAAACAAUGUACAGAUGTT-3’; anti-sense: 5’-AUCUGUAACAUUGGUUCGGCTT-3’) or negative control siRNA (sense: 5’-UUCUCCGAACGUGUACGTCTT-3’; anti-sense: 5’-ACGUACACGUUCCGAGATT-3’) were synthesized (Gene Pharma, China). Transfection was performed by Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Scientific, USA) according to the given protocols. Briefly, RAW264.7 cells were seeded in 6-well plates. Cells at 70% confluent were transfected respectively with RBP-J siRNA or scramble siRNA using RNAiMAX Transfection Reagent. After 24 h of transfection, the transfection medium was replaced by fresh α-MEM medium. Then, qRT-PCR and Western blot were performed to test the efficiency of knockdown after 24 or 48 h.

**Statistical analysis**

All statistical analysis were performed by GraphPad Prism 8.0.2 (GraphPad Software Inc., USA). All data are presented as the mean ± standard deviation (SD). The results were statistically analyzed with Student’s t-test for two-group comparisons and one-way ANOVA with Tukey’s post hoc test for multigroup comparisons. Data with P-values < 0.05 were considered statistically significant.

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**Fig. 1** Effect of AGEs on RAW264.7 macrophages viability. **a** CCK-8 assay for detecting cells proliferation activity. **b-c** Detection of cells apoptosis by flow cytometry. Percentage of cells apoptosis refers to the sum of early apoptosis rate and late apoptosis rate (Q2 + Q3). Error bar represent mean ± SD; *p < 0.05
Results

Effect of AGEs on RAW264.7 macrophages viability

To investigate the effect of AGEs on the proliferation of RAW264.7 cells, 100, 200 and 400 mg/L AGEs were used to treat cells for 12, 24, 36 and 48 h, respectively. The CCK-8 results showed that the proliferation activity of cells treated with 100 mg/L AGEs was similar to that of the control group within 48 h, while the cells viability was predominantly inhibited when the concentration was higher than that of 100 mg/L (Fig. 1a). Additional analysis on the percentage of apoptosis indicated that 200 and 400 mg/L AGEs significantly induced apoptosis, while 100 mg/L AGEs exerted no substantial effects on it (Fig. 1b-c). Inspired by the above results, 100 mg/L AGEs were selected for follow-up experiments to ensure that potential cytotoxicity would not affect our subsequent measurements.

AGEs induce macrophages phenotype polarization to M1

To explore the role of AGEs on the phenotype polarization of RAW264.7 cells, Griess assay was used to detect the content of NO in the supernatant. The results showed that AGEs significantly promoted the release of NO compared with the control group (Fig. 2a). The qRT-PCR results showed that AGEs substantially up-regulated the mRNA expression of the M1 markers such as iNOS, IL-6 and IL-12b, while M2 markers such as Arg-1 and IL-10 were not elevated (Fig. 2b). The Western blot analysis further confirmed that the expression of iNOS protein obviously increased under the stimulation of AGEs compared with the control group (Fig. 2c).

Fig. 2 AGEs induce macrophages phenotype polarization to M1. a Griess assay for NO generation. b qRT-PCR analysis of M1/M2-related markers expression (M1: iNOS, IL-6 and IL-12; M2: Arg-1 and IL-10). LPS and IL-4 treated groups as positive controls. c Western blot analysis of iNOS expression. GAPDH was detected as control. d Flow cytometry analysis of CD86 and CD206 expression. Error bar represent mean ± SD; *p < 0.05
To further validate our findings, flow cytometry was used to analyze the expression of CD86 and CD206, the specific antigen markers of M1 and M2 macrophages. As expected, we discovered that the expression of CD86 increased and CD206 decreased in AGEs-treated macrophages (Fig. 2d).

**M1 macrophages tend to differentiate into osteoclasts mediated by AGEs**

To compare the differentiation capacity of M1 and M2 macrophages into osteoclasts, we used RANKL and M-CSF to stimulate M1 and M2 macrophages. The qRT-PCR results showed that M1 macrophages were more likely to express osteoclast-related genes such as NFATc-1, TRAF-6, RANK, TRAP and CTSK than M2 macrophages (Fig. 3a), suggesting that M1 macrophages have stronger ability to differentiate into osteoclasts.

We further examined the effect of AGEs on the osteoclast differentiation ability of M1 macrophages. Compared with the control group, adding AGEs at the same time of inducing osteoclasts differentiation could significantly up-regulate the mRNA expression of osteoclast-related genes (Fig. 3b). To further establish that AGEs did mediate the differentiation of macrophages into osteoclasts, TRAP staining was performed. In Fig. 3c-d, we noted that the number of mature osteoclasts in the AGEs intervention group was approximately 3 times as high as in the control group on the 6th day, which further verified our findings.

**AGEs activate notch signaling pathway in macrophages**

As shown in Fig. 4a, the mRNA levels of ligand Jagged1, receptor Notch1 and downstream target gene Hey1 of Notch signaling pathway were up-regulated in AGEs-treated group, suggesting that the Notch signaling pathway may be activated. To further investigate this, total cellular proteins were extracted and analyzed by Western blot. The results showed that the level of Notch1 protein was up-regulated, while the level of Notch1 intracellular domain NICD1 was decreased (Fig. 4b). Moreover, we extracted cytoplasmic and nuclear proteins respectively for Western blot analysis, and were surprised to observe that NICD1 protein almost did not exist in the cytoplasm, but was highly expressed in the nucleus. Meanwhile, compared with the control group, the expression of nuclear NICD1 was significantly increased in the AGEs group (Fig. 4c-d), indicating that AGEs promoted the nuclear translocation of NICD1.

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**Fig. 3** M1 macrophages tend to differentiate into osteoclasts mediated by AGEs. **a** qRT-PCR analysis of osteoclast-related markers expression (NFATc-1, TRAF-6, RANK, TRAP and CTSK) to compare the difference of osteoclastogenesis of M1/M2. **b** qRT-PCR analysis of osteoclast-related markers expression in M1 macrophages treated with AGEs. **c-d** TRAP staining for osteoclastogenesis of M1 macrophages treated with AGEs in high power field (×200) and the number of mature osteoclasts (marked with the black arrow). Error bar represent mean ± SD; *p < 0.05
of NICD1 to RBP-J after entering the nucleus. The silencing efficiency of siRNA fragments was verified by qRT-PCR and Western blot analysis (Fig. 6a-c). The results showed that AGEs induced M1 polarization effect was significantly alleviated after partial deletion of RBP-J, which was characterized by the declined mRNA and protein expression of M1-related genes (Fig. 6d-e).

Discussion

AGEs, formed by the body metabolites, reducing sugars and proteins under the action of non-enzymatic glycosylation (Maillard reaction), have been revealed to accumulate during aging or the progression of diabetes, which further promote inflammation and cause damage to the body [1, 21]. It has been explored for a long time whether macrophages tend to become inflammatory in response to AGEs and whether M1 macrophages play a dominant role in this process. Reports have suggested that differential NO metabolism is a reliable marker to distinguish the two activation states of macrophages. The high expression of iNOS in M1

Notch inhibitor DAPT attenuates AGEs-mediated M1 polarization in macrophages

DAPT, a γ-secretase inhibitor, was used to block the transduction of Notch signaling pathway.

After Notch signaling pathway was blocked by DAPT, the mRNA levels of Hey1, iNOS, IL-6 and IL-12b enhanced by AGEs were significantly down-regulated (Fig. 5a), and the expression of iNOS protein was also decreased to a certain extent compared with the AGEs-induced group (Fig. 5b). In addition, the flow cytometry analysis revealed that DAPT could also dramatically reverse the increase of M1 marker antigen CD86 and the decrease of M2 marker antigen CD206 caused by AGEs (Fig. 5c).

Knockdown of RBP-J impairs the effect of AGEs on M1 polarization in macrophages

To further verify that Notch signaling pathway was involved in AGEs-mediated macrophages M1 polarization, we designed siRNA interference fragments of the recombinant binding protein (RBP-J) to interfere with the binding process of NICD1 to RBP-J after entering the nucleus. The silencing efficiency of siRNA fragments was verified by qRT-PCR and Western blot analysis (Fig. 6a-c). The results showed that AGEs induced M1 polarization effect was significantly alleviated after partial deletion of RBP-J, which was characterized by the declined mRNA and protein expression of M1-related genes (Fig. 6d-e).
capacity of macrophages. Considering that the clearance of AGEs in vivo mainly depends on the phagocytosis of mononuclear macrophages, we suspected that there might be a potential balance between the AGEs-induced inflammation and the elimination of AGEs by macrophages. The concentration and heterogeneity of AGEs, the types of activated AGEs receptors, and the capacity of macrophages to eliminate excess AGEs may jointly determine the fate of macrophages.

Our research has demonstrated that AGEs have the inflammatory signaling capacity. Interestingly, we observed that under the induction of RANKL and M-CSF, proinflammatory macrophages were prone to highly express osteoclast-related markers, such as NFATc-1, TRAF-6, RANK, TRAP and CTSK, suggesting that M1 macrophages may drive osteoclastogenesis by releasing inflammatory signals. In addition, AGEs are associated with some bone metabolism-related diseases. Guo et al. found that AGEs were involved in the regulation of senile osteoporosis. Cheng et al. reported that diabetes associated osteoporosis could be caused by AGEs. We unexpectedly discovered that AGEs enhanced the differentiation of M1 macrophages into osteoclasts. These evidences suggest that AGEs-mediated macrophages proinflammatory M1 macrophages is involved in the NO synthesis. In the current study, we demonstrated that AGEs induced high expression of iNOS in RAW264.7 macrophages and release of large amounts of NO, indicating that AGEs may promote macrophages to polarize to M1. Macrophages polarization is generally regarded as an innate immune regulatory pathway to explain the pathogenesis of some diseases, as macrophages can secrete various cytokines in response to diverse pathological microenvironments. In this study, AGEs stimulated macrophages to produce a great quantity of pro-inflammatory cytokines such as iNOS, IL-6 and IL-12b, which is probably associated with accelerating the development of AGEs-mediated diabetes or other degenerative diseases.

Recently it has been confirmed that AGEs Receptors of macrophages may play an essential role in the regulation of AGEs-mediated M1 polarization, including receptor for advanced glycation end products (RAGE), galectin-3, and CD36. To date, most studies believe that RAGE is involved in AGEs-mediated macrophages M1 polarization. Galectin-3 expressed in CD206+ macrophages (M2) was shown to enhance the phagocytosis of apoptotic cells and cellular debris by macrophages. Similarly, the expression of CD36 could elevate the phagocytic capacity of macrophages. Considering that the clearance of AGEs in vivo mainly depends on the phagocytosis of mononuclear macrophages, we suspected that there might be a potential balance between the AGEs-induced inflammation and the elimination of AGEs by macrophages. The concentration and heterogeneity of AGEs, the types of activated AGEs receptors, and the capacity of macrophages to eliminate excess AGEs may jointly determine the fate of macrophages.

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AGEs. DAPT is one of the commonly used inhibitors of the Notch signaling pathway, whose target is γ-secretase, a proteolytic enzyme that can dissociate NICD from Notch\[32\]. RBP-J is a DNA binding component of the transcriptional complex regulated by typical Notch signal transduction. When the Notch receptor is activated, RBP-J can be bound by NICD translocated into the nucleus, which interacts with a transcriptional activation complex containing Mastermind (MAML)-like protein and histone acetylase p300, resulting in the transcriptional activation of the Notch target genes\[30, 33\]. It has been reported that knockdown of RBP-J can effectively impede the transduction of Notch signaling pathway and inhibit the occurrence of macrophages M1 polarization\[34–36\]. Therefore, in the current study, we used DAPT and Si-RBP-J to block the above two key links respectively, and then found that the M1 polarization mediated by AGEs was weakened to varying degrees, suggesting that the Notch signaling pathway is involved in the process of macrophages polarization mediated by AGEs. Inspired by the above results, we suspect that efficiently blocking the Notch signal transduction can be conducive to delaying the pathogenesis of related degenerative diseases through reversing the macrophages M1 polarization induced by AGEs.

Our study may lead to a better understanding of AGEs-mediated bone immunomodulatory mechanisms and reveal the potential role of AGEs in macrophages M1 polarization.

Fig. 6 Knockdown of RBP-J impairs the effect of AGEs on M1 polarization in macrophages. a-c qRT-PCR and Western blot analysis of RBP-J expression to detect the silencing efficiency of siRNA fragments. d qRT-PCR analysis of Hey1, iNOS, IL-6 and IL-12b expression. e Western blot analysis of iNOS expression. GAPDH was detected as control. Error bar represent mean ± SD; *p < 0.05

Recently, some potential mechanisms related to AGEs-mediated macrophages polarization have been uncovered, such as certain canonical pathways involving MAPK and TLRs signaling\[28, 29\]. In this study, whereas, Notch signaling pathway was revealed to mediate AGEs-induced macrophages M1 polarization, as well. The classical activation process of Notch signaling pathway is as follows: after Notch receptor activation by the ligand of an adjacent cell, Notch intracellular domain (NICD) is released from the inside of the cell membrane by γ-secretase and transfer into the nucleus, where it binds to the DNA binding protein RBP-J (also known as CSL or CBF1), eventually resulting in the activation of downstream Notch target genes\[30\]. In the current study, AGEs were demonstrated to elevate the mRNA or protein levels of ligand Jagged1, receptor Notch1, transcriptional cofactor RBP-J and downstream target gene Hey1 of Notch signaling pathway, suggesting that the Notch signaling cascade was activated by AGEs. Interestingly, we found the expression of NICD1, a key indicator of the activation of Notch signaling pathway\[31\], increased in the nucleus but not in the cytoplasm, strongly proving that AGEs promoted the entry of NICD1 into the nucleus.

Furthermore, pathway inhibitors and siRNA were applied to investigate whether the activation of Notch signals is related to the macrophages M1 polarization caused by AGEs. DAPT is one of the commonly used inhibitors of the Notch signaling pathway, whose target is γ-secretase, a proteolytic enzyme that can dissociate NICD from Notch\[32\]. RBP-J is a DNA binding component of the transcriptional complex regulated by typical Notch signal transduction. When the Notch receptor is activated, RBP-J can be bound by NICD translocated into the nucleus, which interacts with a transcriptional activation complex containing Mastermind (MAML)-like protein and histone acetylase p300, resulting in the transcriptional activation of the Notch target genes\[30, 33\]. It has been reported that knockdown of RBP-J can effectively impede the transduction of Notch signaling pathway and inhibit the occurrence of macrophages M1 polarization\[34–36\]. Therefore, in the current study, we used DAPT and Si-RBP-J to block the above two key links respectively, and then found that the M1 polarization mediated by AGEs was weakened to varying degrees, suggesting that the Notch signaling pathway is involved in the process of macrophages polarization mediated by AGEs. Inspired by the above results, we suspect that efficiently blocking the Notch signal transduction can be conducive to delaying the pathogenesis of related degenerative diseases through reversing the macrophages M1 polarization induced by AGEs.

Our study may lead to a better understanding of AGEs-mediated bone immunomodulatory mechanisms and reveal the potential role of AGEs in macrophages M1 polarization.
and osteoclastogenesis. Moreover, since we demonstrated the efficacy of blocking Notch signaling pathway in preventing M1 polarization, Notch signaling pathway may represent a new target for improving AGES-mediated degenerative diseases such as senile or diabetic osteoporosis.

**Author contribution** All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Hao Tan, Xiaqian Ding, and Leilei Zheng. The first draft of the manuscript was written by Hao Tan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data availability statement** The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethics approval** We declare that this study only used purchased murine cell lines and did not involve experimental animals or human participants, so no special ethical approval is required.

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