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 both play essential roles in degenerative bone diseases caused by aging or diabetes, such as senile or diabetic osteoporosis. Here, we aimed to understand the involvement and potential mechanism of AGEs in macrophages polarization and osteoclastogenesis. We found that RAW264.7 macrophages treated with AGEs highly expressed M1-associated genes and surface antigen markers CD86, and released a large amount of NO to the extracellular environment. Through the detection of osteoclast-related markers and TRAP staining, we revealed that the osteoclastogenesis of M1 macrophages could be markedly enhanced by AGEs. To explore the potential mechanisms of AGEs-mediated M1 polarization, we first demonstrated that AGEs effectively activated the transduction of Notch signaling pathway, including nuclear translocation of NICD1. Subsequently, it was observed that the M1 polarization effects induced by AGEs were significantly mitigated, when γ-secretase inhibitor DAPT and siRNA targeting silencing RBP-J were applied to block the signal transduction of Notch. In conclusion, our findings revealed a series of phenomena that AGEs induce macrophage M1 polarization and enhance its osteoclastogenesis ability, and Notch/NICD/RBP-J signaling axis is involved in the regulation of this polarization process.

1. 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. The formation of AGEs in vivo is inevitable, and its clearance mainly depends on the phagocytosis of mononuclear macrophages.[1] Exogenous AGEs are mostly found in foods with high carbohydrate and fat content, especially when these foods are heat-treated [2]. AGEs are continuously produced and accumulated in the body, no matter in physiological or pathological conditions, especially in the process of aging or poor glycometabolism in diabetics [3, 4]. It is noteworthy that the accumulation of AGEs will further quicken the aging of human body and lead to the occurrence of some chronic degenerative diseases, such as diabetes and senile osteoporosis [5, 6]. Thus, the focused study of AGEs may help to develop much-needed new therapeutic strategies for these diseases.

Macrophages, as the precursor of osteoclasts, can differentiate into osteoclasts through the classical RANK-RANKL pathway, which has been successfully simulated in previous studies [7, 8]. In addition, macrophages are also one of the innate immune cells characterized by high plasticity. It is well known that macrophages are mainly classified as classically activated macrophages (M1) and alternatively activated macrophages (M2) [9]. 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 [10]. However, there are still some controversies about which kind of macrophages are more likely to differentiate into osteoclasts suggesting that it is of great significance for us to explore the relationship between macrophage polarization and osteoclasts.

Increasing facts have proved 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 [11, 12]. However, according to another research, AGEs were reported to weaken the activation of inflammasomes and inhibit the M1 polarization of macrophages induced by lipopolysaccharide [13]. Therefore, the specific effect of AGEs on macrophages polarization is still inconclusive, and its latent mechanisms also remain obscure. At the same time, exploring the potential relationship between AGEs-mediated macrophages polarization and osteoclasts differentiation also provides a new direction for researching the imbalance of bone metabolism caused by the direct or indirect action of AGEs in aging or diabetes.

Notch signaling pathway, characterized by a high degree of conservatism, widely participates in the development and differentiation of organs, tissues and cells, especially plays a critical role in regulating the function of immune system cells such as monocytes, macrophages and lymphocytes [14]. 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) [15, 16]. Notch signal is also considered to be a key regulator of the biological function of macrophages [17]. Study demonstrated that Notch signal on macrophages can regulate the macrophages differentiate into M1, and activate macrophages to release more cytokines and chemokines for promoting the inflammatory response [18]. Although Notch signal has been confirmed to regulate classical macrophages polarization, whether Notch signaling pathway is involved in macrophages polarization mediated by AGEs still remains unreported.

Based on the above background, we propose the following hypothesis: AGEs can effectively mediate the polarization and osteoclastogenesis of macrophages, and Notch signaling pathway may play a role in it.

2. Materials And Methods

2.1 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 100 U/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), 1μg/ml LPS (Solarbio, China) and 20ng/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. The first 3 days of osteoclasts differentiation were considered to be the early stage, and the last 3 days were considered to be the late stage. Three repeat holes were set for each sample.

2.2 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，RAW 264.7 cells were seeded in a 96-well plate at a density of 5×10^3 /well . After 24h, the medium was replaced with fresh α-MEM medium containing AGEs at concentrations of 0, 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 0, 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 7.6.1.

2.3 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 100mg/L AGEs for 24 h, the cell supernatant of each group was extracted and added to a 96-well plate at 50 μl/well. According to the instructions, 50 μl of Griess Reagent I and 50 μl 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.

2.4 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 20 μl 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 an internal control and all reactions were performed in triplicate. The sequences of the primers (Sangon Biotech, China) are listed in Table 1.

2.5 Western blot analysis

Cells were lysed with RIPA buffer (Santa Cruz Biotechnology, USA) containing 1 mM phenylmethylsulfonyl fluoride (Beyotime, China) after washed by cold PBS three times. The total protein concentration was quantified with an enhanced bicinchoninic acid (BCA) protein analysis kit (Beyotime, China). For western blot analysis, 30 μg of protein extracts were loaded onto gels respectively and

separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, China). Then these proteins were transferred to a polyvinylidene fluoride membrane, after which the membrane was blocked with fat-free milk buffer for 2 h and blotted overnight at 4°C with primary antibodies (1:1000, Cell Signaling Technology, 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).

2.6 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 500 μl 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 cytometer (BD Influx, USA). The data were statistically analyzed by FlowJo 7.6.1.

2.7 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 with 4% paraformaldehyde at room temperature for 20 minutes 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).

2.8 Treatment with DAPT

DAPT (Abmole, USA), as a new type of γ-secretase inhibitor, is usually used as an inhibitor of the Notch signaling pathway. While treating the cells with different stimuli, 5 μmol/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.

2.9 Gene interference with RBP-J siRNA

Small interfering RNA (siRNA) targeting RBP-J (sense: 5'-GCCGAAACAAUGUACAGAUTT-3'; anti-sense: 5'-AUCUGUACAUUGUUUCGGCTT-3') or negative control siRNA (sense: 5'-UUCUCCGAACGUGUCACGUTT-3'; anti-sense: 5'-ACGUGACACGUUCGGAGAATT-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 48h.

2.10 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.

3. Results

3.1 Effect of AGEs on Raw 264.7 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 100mg/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 100mg/L (Fig. 1a). Additional analysis on the percentage of apoptosis indicated that 200 and 400mg/L AGEs significantly induced apoptosis, while 100mg/L AGEs exerted no substantial effects on it (Fig. 1b-c). Inspired by the above results, 100mg/L AGEs were selected for follow-up experiments to ensure that potential cytotoxicity did not affect our subsequent measurements.

3.2 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). Furthermore, 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.2a). Western blot analysis further confirmed that compared with the control group, the expression of iNOS protein obviously increased under the stimulation of AGEs (Fig. 2d-e). To further validate our findings，flow cytometry was used to analyze the expression of CD86, the surface antigen marker of M1 macrophages. To be expected, we discovered elevated positive expression rate of CD86 on macrophages in response to AGEs (Fig. 2b-c). All the above evidences fully demonstrate that AGEs induce macrophage phenotype polarization to M1.

3.3 M1 macrophages tend to differentiate into osteoclasts mediated by AGEs

To compare the difference between the ability of M1 and M2 macrophages to differentiate into osteoclasts, we used RANKL and M-CSF to induce 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 a 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 and the later intervention group, the addition of AGEs in the early stage of osteoclasts induction could significantly up-regulate the mRNA expression of osteoclast-related genes (Fig. 3b). To further establish that AGEs indeed mediate the differentiation of macrophages into osteoclasts, we performed the TRAP staining. In Figure 3c-d, we noticed that the number of mature osteoclasts in the AGEs early intervention group was about 3 times higher than that in the control group on the 6th day, which further verified our findings. All the above results illustrate that AGEs can significantly enhance the ability of M1 macrophages to differentiate into osteoclasts.

3.4 AGEs activate Notch signaling pathway in macrophages

As shown in figure 4, the mRNA levels of ligand Jagged1, receptor Notch1 and downstream target gene Hey1 of Notch signaling pathway were observed to be up-regulated in AGEs-treated group, suggesting that Notch signaling pathway may be activated (Fig 4a). To further investigate this, total cellular proteins were extracted and analyzed by Western blot. The results showed that the protein level of Notch1 was also up-regulated, while the level of Notch1 intracellular domain NICD1 decreased (Fig. 4b). Moreover, we extracted cytoplasmic and nuclear proteins respectively and observed the increased expression of NICD1 protein in the nucleus by Western blot analysis (Fig. 4c-d). These results confirm that AGEs activate Notch signaling pathway in Raw264.7 cells and promote nuclear translocation of NICD1, thus activating the expression of downstream target genes.

3.5 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 the blockade of Notch signaling pathway by DAPT, the expression of M1-related genes iNOS, IL-6 and IL-12b enhanced by AGEs was significantly down-regulated at mRNA level (Fig. 5a), and iNOS protein expression level was also retarded to some extent compared with AGEs-induced group (Fig. 5d-e). In addition, flow cytometry revealed that DAPT could dramatically reduce the increase of M1 surface marker antigen CD86 induced by AGEs, as well (Fig. 5b-c). These results demonstrate that blocking Notch signal pathway with γ-secretase inhibitor DAPT can effectively attenuate AGEs-mediated M1 polarization in macrophages.

3.6 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 for 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 (Fig. 6a-c). Further results showed that after partial knockdown of RBP-J, the increased mRNA expression of M1 markers induced by AGEs was

decreased (Fig. 6d), and the up-regulated iNOS protein expression was also down-regulated (Fig. 6e-f). Taken together, these results illustrate that knockdown of RBP-J can impair the effect of AGEs on macrophages M1 polarization to a certain extent.

4. Discussion

AGEs, formed by the body metabolites, reducing sugars and proteins under the action of non-enzymatic glycosylation (Maillard reaction), have been proved to accumulate in the process of aging or diabetes, which further promote inflammation and cause damage to the body [1, 19]. Under the action of AGEs, whether macrophages tend to inflammatory state and M1 macrophages play a leading role in it have been explored for a long time. 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 macrophages is involved in the process of NO synthesis [20, 21]. In 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 induce macrophages to polarize to M1. Moreover, the up-regulated inflammation-related factors IL-6, IL-12b further confirmed the inflammatory induction effect of AGEs on macrophages, which accorded with previous reports [10, 22]. Flow cytometry is usually used as a criterion for cell typing, and CD86 is generally regarded as the surface marker antigen of M1 macrophages in previous studies [23, 24]. Here, our study further verified the conclusion that AGEs can induce M1 polarization by observing a profound increase in the number of CD86 positive cells. Based on the above evidences, we infer that AGEs can induce macrophages M1 polarization and play a critical role in inflammatory regulation, which may be one of the potential mechanisms or causes of degenerative diseases caused by AGEs.

As the precursor of osteoclasts, macrophages can differentiate into osteoclasts and gradually mature, which is a dynamic and complex process [25, 26]. Here, our study first observed that M1 macrophages prefer to highly express the above osteoclast-related markers, proving that M1 macrophages have greater osteoclastogenesis potential, which is consistent with the research of Huang et al [27]. To date, some reports have been put to confirm that AGEs are related to osteoclasts metabolism, but its specific role in regulating bone resorption is not clear [28, 29]. We then examined the role of AGEs in the differentiation of M1 macrophages into osteoclasts. Since it is a dynamic process for macrophages to differentiate into osteoclasts and mature, our team previously explored the process of osteoclasts differentiation and found that mature multinucleated osteoclasts began to appear only on the third day of induction. Hence, in this study, the process of osteoclasts differentiation is divided into early stage and later stage according to the boundary of 3 days. Subsequently, we found that in the early stage, AGEs profoundly promoted the occurrence of osteoclasts resorption, which was characterized by increased expression of osteoclast-related marker genes and the number of TRAP positive cells, while no significant change was observed in the later stage group. Based on the phenomena, it can be inferred that AGEs can enhance the ability of M1 macrophages to differentiate into osteoclasts, which mainly occurs in the first 3 days of osteoclasts differentiation, when nuclear fusion has not yet occurred.

After confirming that AGEs can induce macrophages M1 polarization and ultimately affect its ability to differentiate into osteoclasts, we attempted to explore the potential mechanism of AGEs-induced polarization. It is known that Notch signaling pathway is involved in regulating the development, differentiation and maturation of macrophages and other immune cells [14, 30]. The classical activation process of Notch signaling pathway is: after Notch receptor activation by ligand of adjacent cell, Notch intracellular domain (NICD) is released from the inside of the cell membrane by γ-secretase and transferred 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 classical Notch target genes [31]. Here, we found that macrophages highly expressed ligand Jagged1, receptor Notch1 and downstream target gene Hey1 of Notch signaling pathway under the action of AGEs. Besides, the nuclear translocation of NICD is considered to be a key indicator of the activation of Notch signaling pathway [32]. For this reason, we detected that NICD1 mainly existed in the nuclear protein rather than in the cytoplasm, and the expression of NICD1 in the nucleus was significantly up-regulated by AGEs. Such favorable results fully prove that Notch signaling pathway can be effectively activated by AGEs.

Next, we aimed to investigate whether the activation of Notch signal is related to M1 polarization of macrophages caused by AGEs. As a new type of γ-secretase inhibitor, DAPT is one of the commonly used inhibitors of Notch signaling pathway, which has a strong blocking effect on the transduction of Notch signal [33]. It should be noted that the target of DAPT is γ-secretase, a proteolytic enzyme that can dissociate NICD from Notch [34]. Accordingly, the application of DAPT was adopted as one of the methods to obstruct the Notch signal activation induced by AGEs in this study. As a result, we found that the M1 polarization of macrophages was markedly reversed after such treatment, suggesting that Notch signaling pathway may be involved in the process of macrophages polarization mediated by AGEs.

Reports have suggested that knockdown of RBP-J can effectively impede the transduction of Notch signaling pathway and inhibit the occurrence of macrophages M1 polarization [22, 35, 36]. 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 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 [31, 37]. Here we found that AGEs could not induce the high expression of intracellular RBP-J, but when RBP-J was silenced with specific siRNA fragments, the M1 polarization effect induced by AGEs was partially weakened, which may be due to the artificial blocking of the effective binding between NICD and RBP-J in the nucleus.

In summary, our findings revealed a series of phenomena that AGEs induce macrophages M1 polarization and promote osteoclasts differentiation, and provided a potential connection between Notch/NICD/RBP-J signaling axis and the M1 polarization of macrophages mediated by AGEs (Fig. 7).

Declarations

Author contribution All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Hao Tan, Xiaoqian 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.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethics approval This study was approved by the ethics committee of Chongqing Medical University.

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Tables

Table 1. Primers for Quantitative real-time PCR

Gene(mouse)	Forward Primer (5’ to 3’)	Reverse Primer (5’ to 3’)
iNOS	ACTCAGCCAAGCCCTCACCTAC	TCCAATCTCTGCCTATCCGTCTCG
IL-6	CTTCTTGGGACTGATGCTGGTGAC	AGGTCTGTTGGGAGTGGTATCCTC
IL-12b	CATTGAACTGGCGTTGGAAGCAC	GGGCGGGTCTGGTTTGATGATG
Arg-1	GGCAACCTGTGTCCTTTCTCCTG	GGTCTACGTCTCGCAAGCCAATG
IL-10	TTCTTTCAAACAAAGGACCAGC	GCAACCCAAGTAACCCTTAAAG
NFATc-1	GGCTGGTCTTCCGAGTTCACATC	GCTGTCTGTGCTCTGCTTCTCC
TRAF-6	AGGAATCACTTGGCACGACACTTG	TGGTCCTGTCTTACTAGGCGACTC
RANK	AGCCTCCGAGCAGAACTGACTC	CTGCCTGTGTAGCCATCTGTTGAG
TRAP	CCACCCTGAGATTTGTGGCT	ACATACCAGGGGATGTTGCG
CTSK	CTGGAGGGCCAACTCAAGA	CCTCTGCATTTAGCTGCCTT
Jagged1	AAAAATCAGGACACACAACTCG	CTGTTTATTTGTCCAGTTCGGG
Notch1	GTGCTGGAAGTATTTTAGCGAC	GTCCTTGCAGTACTGGTCATAC
RBP-J	TCCACCAGCCTTACCTTCACCTAC	TTTGACTCATTGGAGGGCACTTGG
Hey1	CTATGGACTATCGGAGTTTGGGGTTTC	GGGATGCGTAGTTGTTGAGATGGG
GAPDH	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC

Figures

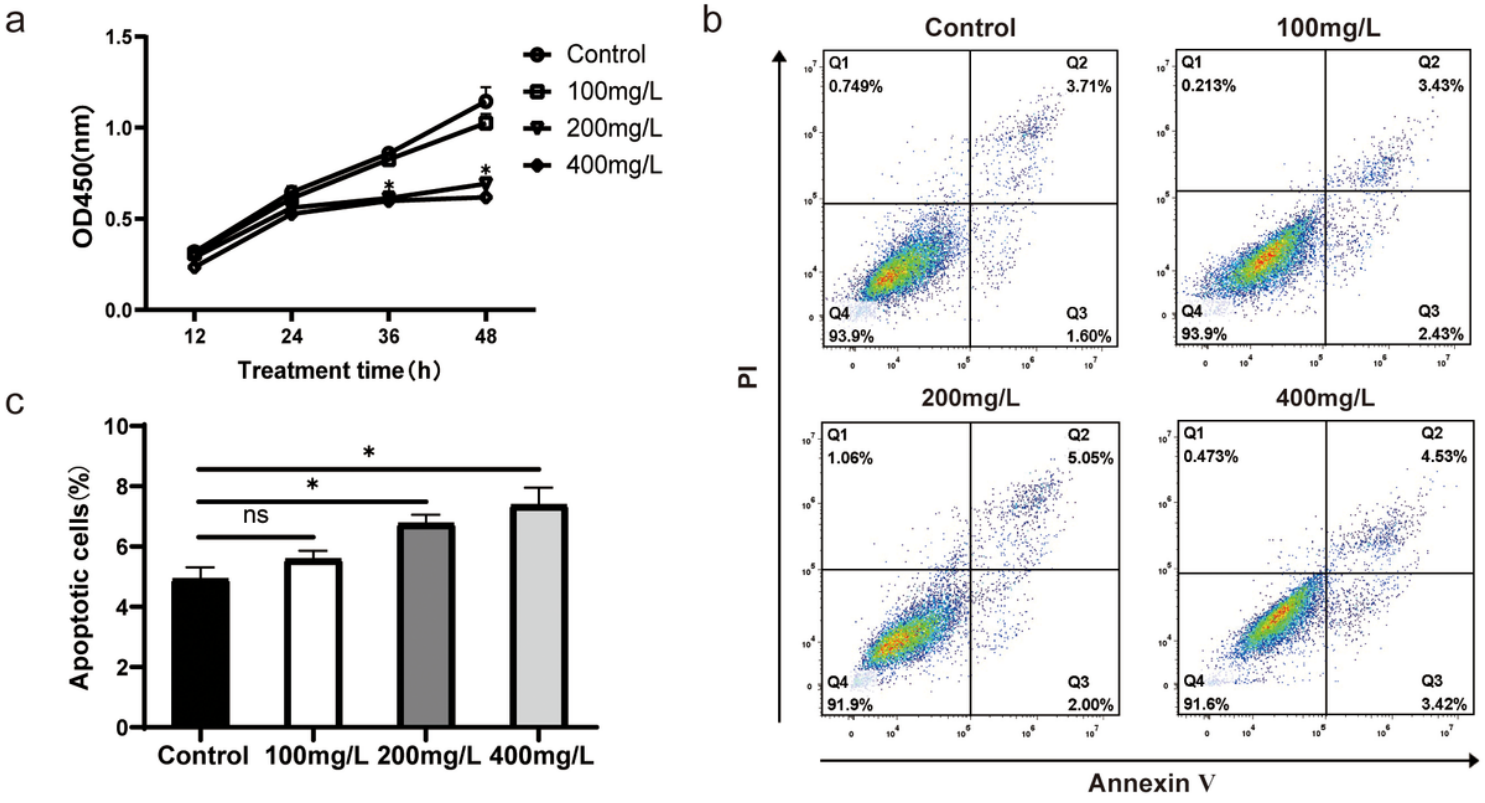

Figure 1

Effect of AGEs on Raw 264.7 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

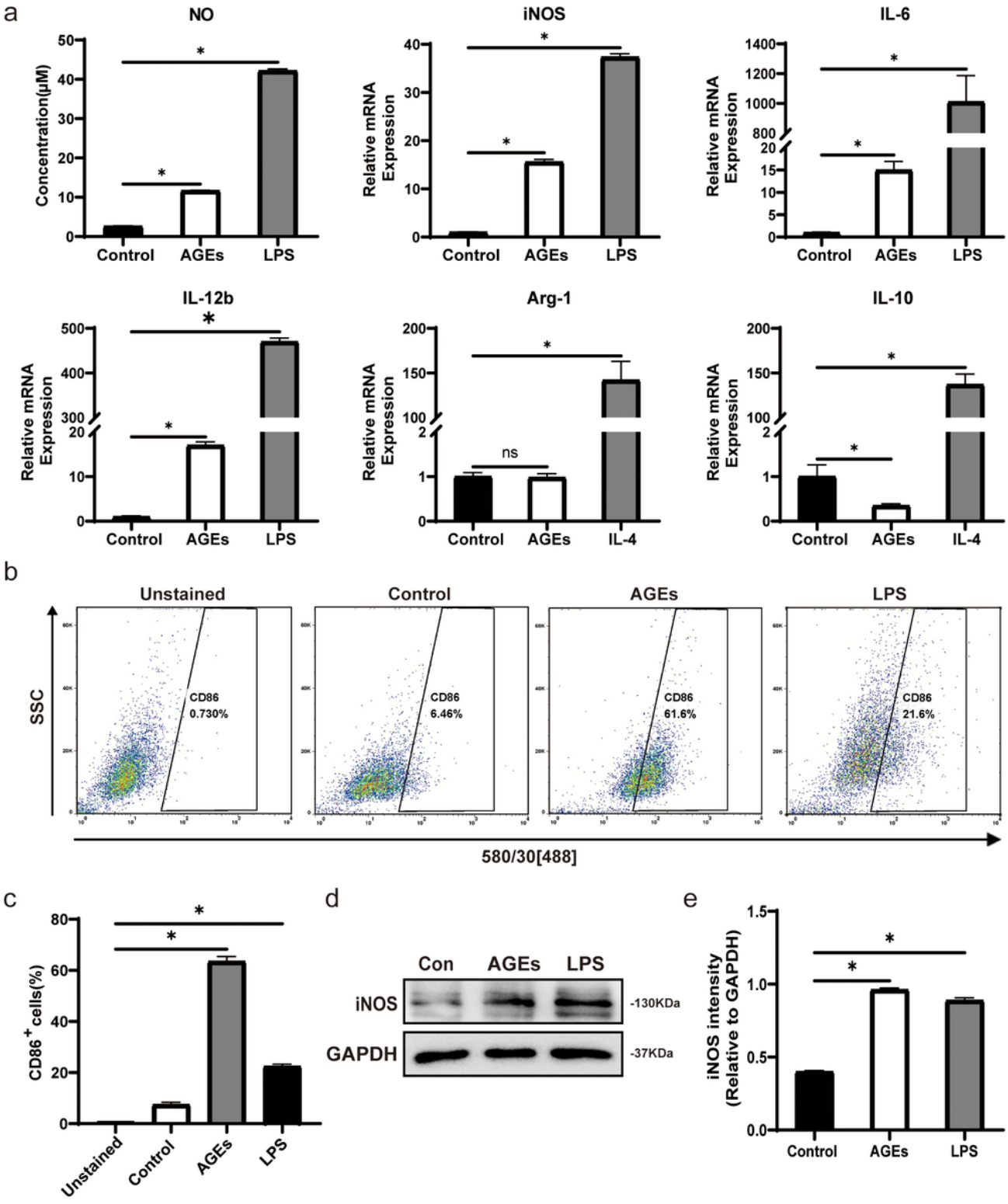

Figure 2

AGEs induce macrophages phenotype polarization to M1. a Griess assay for NO generation and qPCR 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. b-c Flow cytometry analysis of M1 surface antigen CD86 expression. d-e Western blot analysis of iNOS expression based on densitometric statistics. GAPDH was detected as control. Error bar represent mean ± SD; *p<0.05

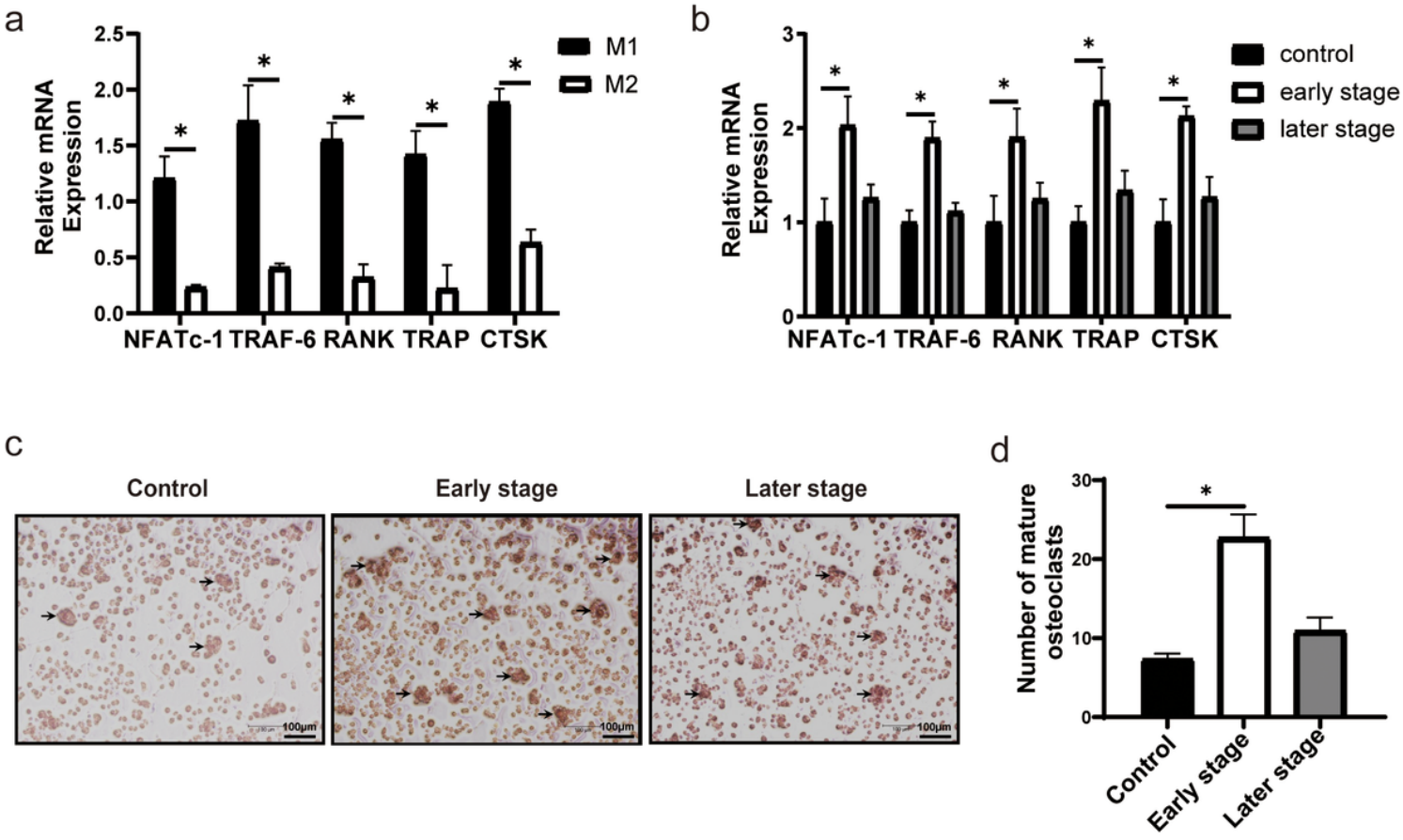

Figure 3

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

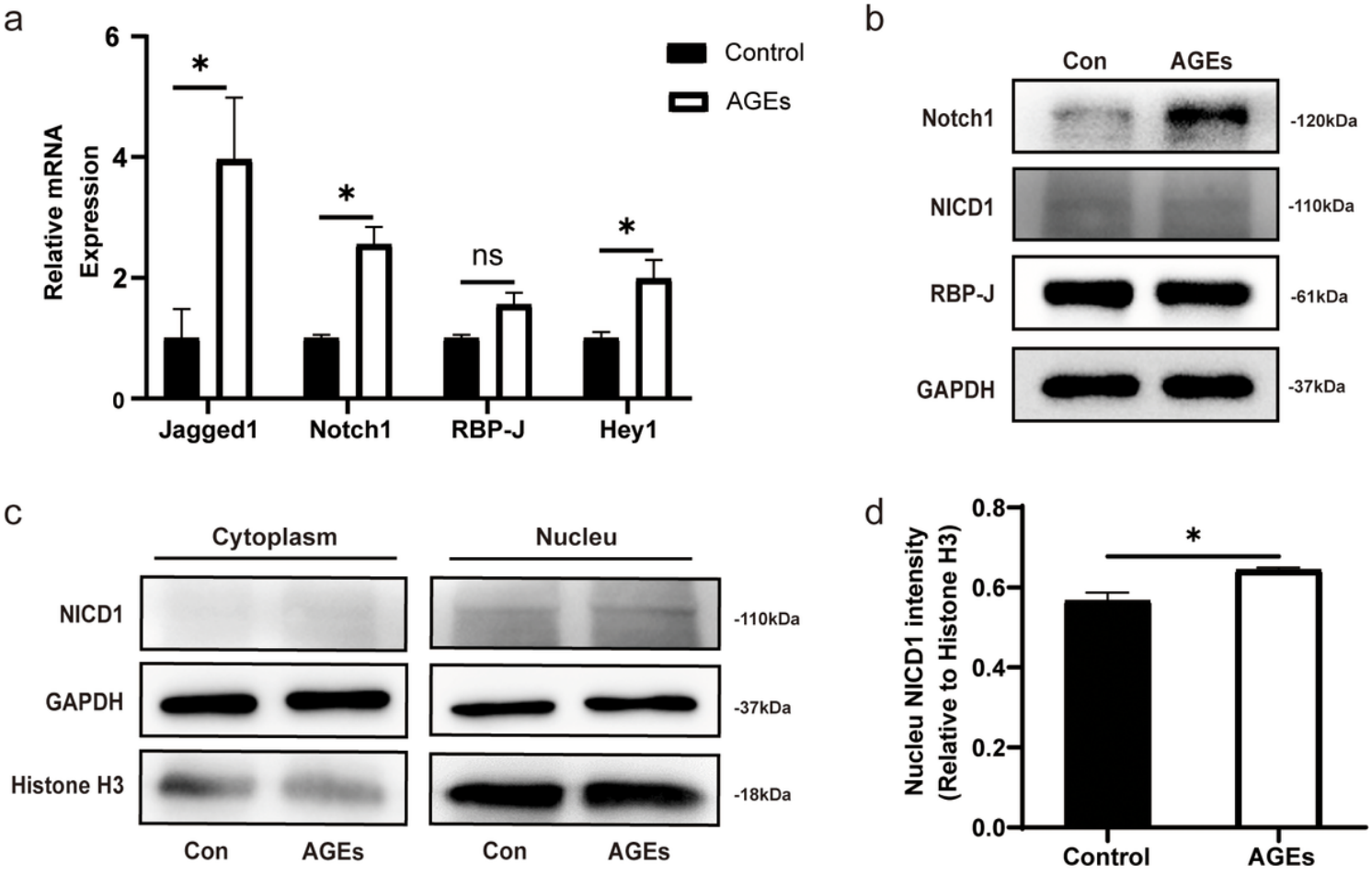

Figure 4

AGEs activate Notch signaling pathways in macrophages. a qRT-PCR analysis of Jagged1, Notch1, RBP-J and Hey1 expression. b Western blot analysis of Notch1, NICD1 and RBP-J. c-d Western blot analysis of NICD1 expression in cytoplasm and nucleus. GAPDH and Histone H3 were detected as control of cytoplasmic and nuclear proteins, respectively. Error bar represent mean ± SD; *p<0.05

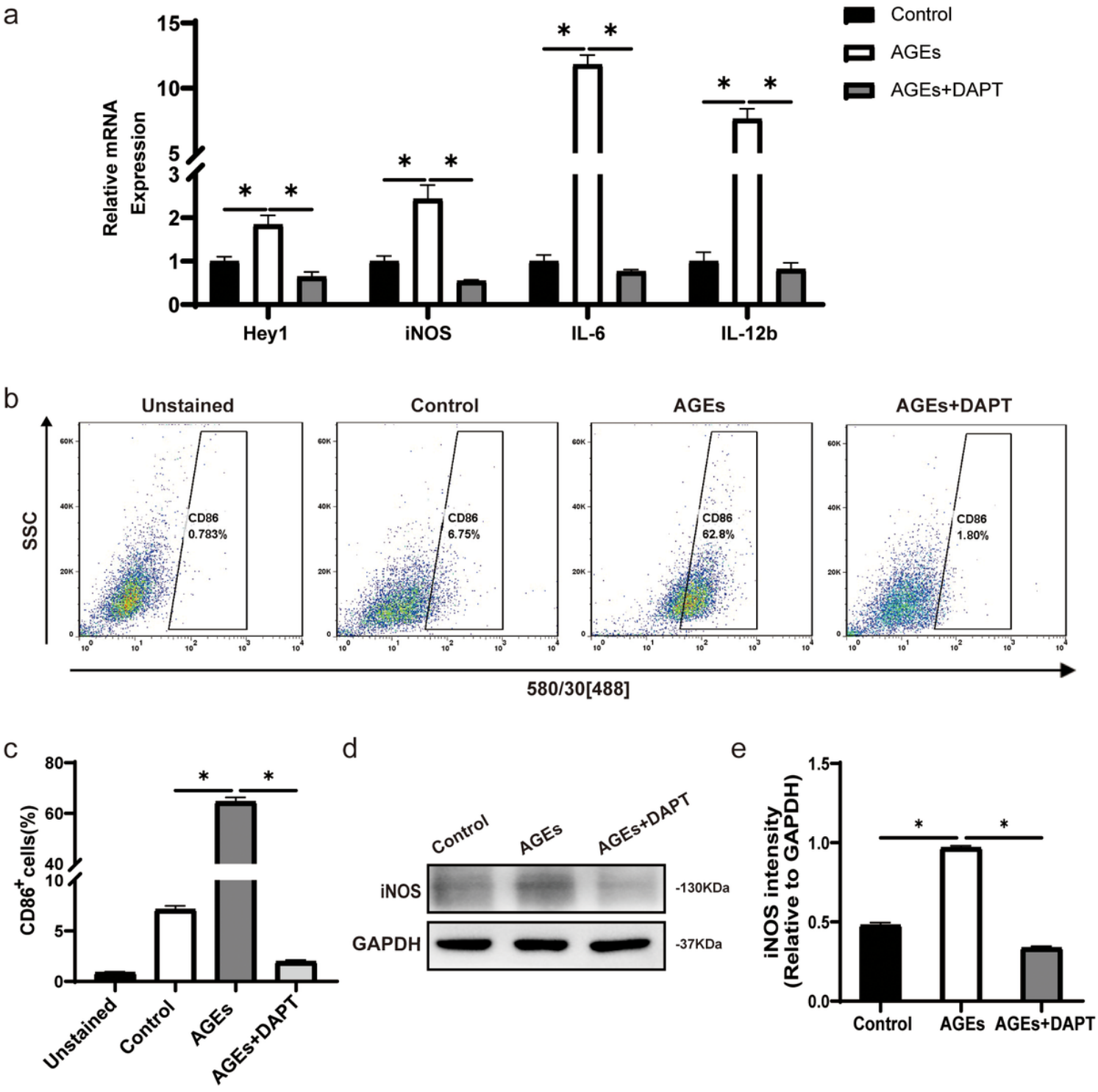

Figure 5

Notch inhibitor DAPT attenuates AGEs-mediated M1 polarization in macrophages. a qRT-PCR analysis of Hey1, iNOS, IL-6 and IL-12b expression. b-c Flow cytometry analysis of M1 surface antigen CD86 expression. d-e Western blot analysis of iNOS expression based on densitometric statistics. GAPDH was detected as control. Error bar represent mean ± SD; *p<0.05

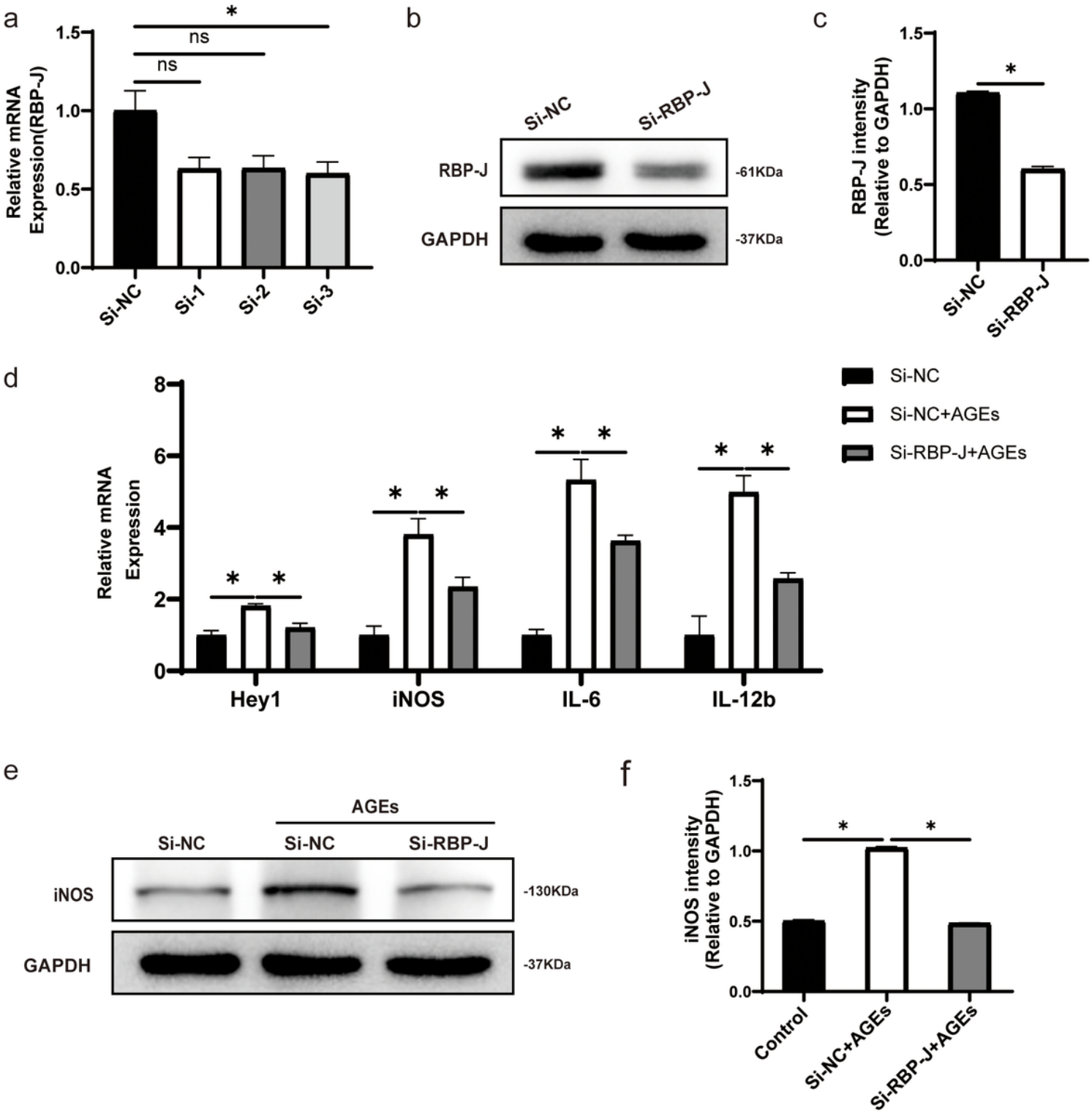

Figure 6

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 qPCR analysis of Hey1, iNOS, IL-6 and IL-12b expression. e-f Western blot analysis of iNOS expression based on densitometric statistics. GAPDH was detected as control. Error bar represent mean ± SD; *p<0.05

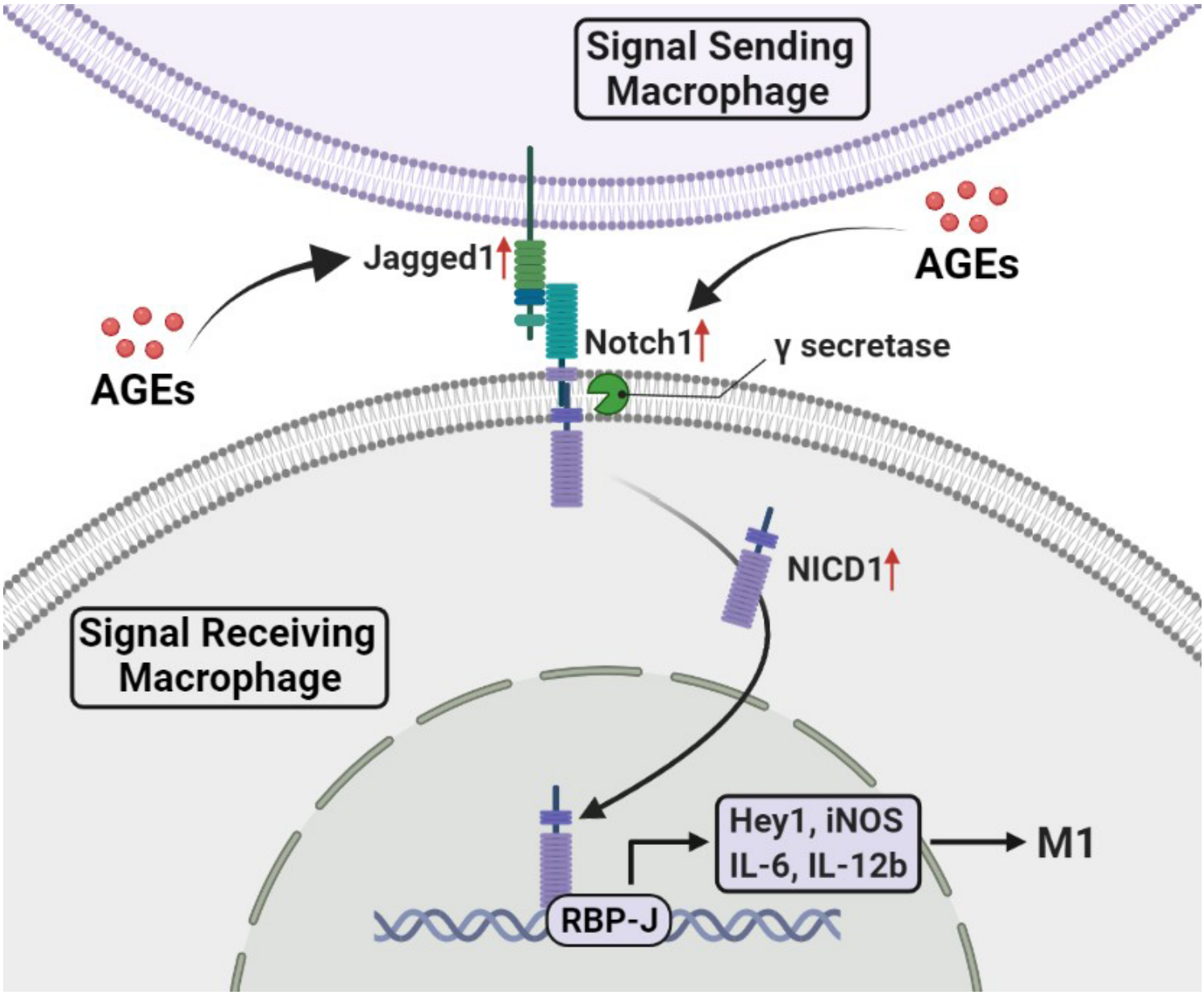

Figure 7

Schematic illustration of Notch/NICD/RBP-J signaling axis regulating AGEs mediated M1 polarization in macrophages. AGEs treatment promotes the expression and binding of Jagged1(ligand) and Notch1(receptor) on macrophage membrane. Under the action of γ-secretase, the Notch1 intracellular domain (NICD1) is dissociated and transported into the nucleus, then combines with RBP-J to transcribe M1-associated genes

Supplementary Files

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