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Original Article

Involvement of the visfatin/toll-like receptor 4 signaling axis in human dental pulp cell senescence: Protection via toll-like receptor 4 blockade

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KEYWORDS
Human dental pulp cells;
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Background: /purpose: Dental pulp plays an important role in the maintenance of tooth homeostasis and repair. The aging of dental pulp affects the functional life of the tooth owing to the senescence of dental pulp cells. Toll-like receptor 4 (TLR4) is involved in regulating cellular senescence in dental pulp. We have recently demonstrated that visfatin induces the senescence of human dental pulp cells (hDPCs). Here, we explored the association of TLR4 with visfatin signaling in cellular senescence in hDPCs.
Materials and methods: mRNA levels were determined using reverse transcription polymerase chain reaction (PCR) and quantitative real time-PCR. Protein levels were determined using immunofluorescence staining and Western blot analysis. Gene silencing was performed using small interfering RNA. The degree of cellular senescence was measured by senescence-associated-β-galactosidase (SA-β-gal) staining. Oxidative stress was determined by measurement of NADP/NADPH levels and intracellular reactive oxygen species (ROS) levels.
Results: Neutralizing anti-TLR4 antibodies or TLR4 inhibitor markedly blocked visfatin-induced hDPCs senescence, as revealed by an increase in the number of SA-β-gal-positive hDPCs and upregulation of p21 and p53 proteins. Moreover, visfatin-induced senescence was associated with excessive ROS production; NADPH consumption; telomere DNA damage induction;

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interleukin (IL)-1β, IL-6, IL-8, cyclooxygenase-2, and tumor necrosis factor-α upregulation; and nuclear factor-κB and mitogen-activated protein kinase activation. All of these alterations were attenuated by TLR4 blockade.
Conclusion: Our findings indicate that TLR4 plays an important role in visfatin-induced senescence of hDPCs and suggest that the visfatin/TLR4 signaling axis can be a novel therapeutic target for the treatment of inflammaging-related diseases, including pulpitis.

Introduction

Dental pulp plays an indispensable role in tooth health.[1,2] Dental pulp tissue is often exposed to the microorganism-rich oral environment via cavities, cracks, or other irritation in the hard tissues of the tooth, resulting in interactions between dental pulp cells and gram-negative bacteria and their components, leading to inflammation.[1–3] Lipopolysaccharide (LPS), a major component present in gram-negative bacteria, has been identified at the infection site in patients with pulpitis, a disease involving inflammation of dental pulp, and is one of major causes of pulpitis.[4–6] Furthermore, stress conditions, such as repeated LPS stimulation, cause dental pulp stem cells to become senescent, leading to aging of dental pulp tissue together with inflammation.[3] These alterations then contribute to impairment of the ability of the dental pulp to protect the teeth.

Toll-like receptor 4 (TLR4) is the first TLR reported in humans and plays a key role in triggering effective innate immune responses.[7–10] Endogenous ligands for TLR4 include saturated fatty acids, fibrinogen, and S100 protein.[9] In addition, TLR4 recognizes pathogens ligands, such as the bacterial endotoxin LPS, which is the primary agonist for TLR4.[7,9,11] Abnormal or excessive activation of TLR4 is responsible for inflammatory diseases, such as sepsis, inflammatory bowel disease, and autoimmune diseases, as well as neurological and vascular diseases, all more common in the elderly, suggesting that TLR4 may have roles in age-related inflammation.[7,9–12]

Visfatin (pre-B-cell colony-enhancing factor [PBEF] or nicotinamide phosphoribosyltransferase [NAMPT]) is an adipocytokine that is mainly secreted from cells but can also be found intracellularly.[13] Visfatin has been implicated in many inflammatory diseases, including cancer, obesity, type 2 diabetes, and cardiovascular diseases.[14,15] Although the roles of visfatin in cellular senescence remain unclear, our previous study showed that visfatin levels increase with age in human dental pulp tissues and that visfatin plays important roles in the senescence of human dental pulp cells (hDPCs) via the nuclear factor (NF)-κB pathway.[16] Moreover, recent studies have shown that visfatin induces TLR4-mediated—NF—κB signaling activation;[17–21] thus, the visfatin/TLR4/NF-κB signaling axis may also contribute to cellular senescence. However, little is known regarding the relationship between visfatin and TLR4 in the pathophysiology of cellular senescence.

In this study, we explored the roles of TLR4 signaling in visfatin-induced cellular senescence in hDPCs and examined the potential mechanisms.

Materials and methods

Antibodies and reagents

The antibodies used in the current study were as follows: visfatin (AdipoGen, San Diego, CA, USA); α-Tubulin (Bioworld, Minneapolis, MN, USA); β-Actin (Abcam, Cambridge, MA, USA); p21, p53, NF-κB p65, TRF-1, and TLR4 (Santa Cruz Biotechnology, Dallas, TX, USA); γH2AX, p-ERK1/2, p-JNK1/2, p-p38, ERK1/2, JNK1/2, p38, IkBα, and IkBα (Cell Signaling Technology, Danvers, MA, USA); Horseradish peroxidase-conjugated IgG (ENZO, Farmingdale, NY, USA); Alexa Fluor® 488-conjugated IgG and Alexa and Fluor® 594-conjugated IgG (Invitrogen, Camarillo, CA, USA). Visfatin protein was purchased from AdipoGen.

Cell culture

A cell line of immortalized human dental pulp cells (hDPCs) were kindly provided by Dr. Takashi Takata at Tokuyama University in Japan.[22] The hDPCs were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA) and 1% antibiotics-antimycotics (Gibco BRL). The cells were grown at 37 °C in a 5% CO_2 atmosphere.

Gene silencing

Gene silencing experiment was performed as previously described.[16] Double-stranded small interfering RNA (siRNA) oligonucleotides were designed against visfatin (5′-CCACCCAACACAAGCAAAGUUUAUUTTdTdT-3′ and 3′-dTdTAAUAAACUUUGCUUGUGUUGGGUGG-5′), and a negative control was purchased from Bioneer (Daejeon, South Korea).

Reverse-transcription quantitative real-time polymerase chain reaction

Total RNA was isolated from hDPCs using TRIzol reagent (Invitrogen). cDNA was synthesized from 2 μg of total RNA with a Reverse Transcription Kit (iNtRON Biotechnology, Sungnam, South Korea). qPCR was performed using an SYBR® Green method (Roche Applied Science, Penzberg Upper Bavaria, Germany). The entire cycling process, including data analysis, took less than 60 min and was monitored using Light Cycler software (version 4.0). The sequences of the oligonucleotide primers for PCR and qPCR were as follows: Visfatin 5′-GGATCCATGAATCCTGCGGCAGAAGC-3′ and 5′-

CTCGAGATGATGTGCTGCTTCCAGTTC-3′; IL-1β, 5′-GGATATG GAGCAACAAGTGG-3′ and 5′-ATGTACCAGTTGGGGAACTG-3′; IL-6, 5′-AGATTCCAAAGATGTAGCCG- 3′ and 5′-TCTTTGCTG CTTTCACACAT-3′; TNF-α, 5′-GAGTGACAAGCCTGTAGCCA-3′ and 5′-GCAATGATCCCAAAGTAGACC-3′; COX-2, 5′-TTCTTTG CCCAGCACTTCAC-3′ and 5′-CTGCTCATCACCCCATTCAG-3′; IL-8, 5′-ATGACTTCCAAGCTGGCCGTG-3′ and 5′-CTCAGCCC TCTTCAAAAACTTC-3′; β-Actin, 5′-GACTACCTCATGAAGATG-3′ and 5′-GATCCACATCTGCTGGAA-3′; GAPDH, 5′-ATCTTCC AGGAGCGAGATCC-3′ and 5′-AGGAGGCATTGCTGATGATC-3′.

Western blot analysis

Western blot analysis was performed as previously described.[16] The protein blots on the membranes were visualized by ECL detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Senescence-associated-β-galactosidase staining

The degree of SA-β-gal activity was measured using a Senescence Cells Histochemical Staining kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's protocol. The cells were photographed under an Olympus-IX71 microscope (Olympus, Tokyo, Japan). Each experiment was performed in duplicate; three separate experiments were carried out for each group.

Immunocytochemistry

Cells cultured on coverslips were fixed in 4% paraformaldehyde/PBS, blocked with 1% bovine serum albumin/PBS, and then labeled first with the appropriate primary antibodies followed by Alexa Fluor 488-conjugated or Alexa Fluor 594-conjugated secondary antibodies. Coverslips were mounted on slides with fluorescent mounting medium containing DAPI (Vector Labs, Burlingame, CA, USA). Cells were analyzed using a LSM900 confocal microscope (ZEISS, Oberkochen, Germany).

Nicotinamide adenine dinucleotide phosphate/nicotinamide dinucleotide phosphate hydrogen assay

NADP/NADPH levels were assessed using a colorimetric NADP/NADPH assay kit (Abcam) following the manufacturer's instructions. Absorbance was measured with a Multimode Plate Reader Victor X3, P (PerkinElmer, Hopkinton, MA, USA) at 450 nm.

Reactive oxygen species measurement

Total intracellular ROS generation was measured using ROS-ID® Total ROS detection kit (Enzo), according to a previously reported protocol. Fluorescence was measured at 490 nm (excitation)/525 nm (emission) using a fluorescent microplate reader (BioTek, Winooski, VT, USA). Intracellular ROS were analyzed using a LSM900 confocal microscope (ZEISS).

Statistical analysis

Data are representative of at least three independent experiments and are presented as mean ± standard deviations. Statistical comparisons between groups were made using one-way analysis of variance followed by Student's t-tests.

Results

Visfain increased cellular senescence in hDPCs

To evaluate the effects of visfatin on the induction of cellular senescence, siRNA was used to knockdown visfatin expression. The levels of visfatin mRNA and protein were decreased in visfatin siRNA-transfected cells (Fig. 1A and B; Figure s1A,B). SA-β-gal staining assays showed a reduction in the fraction of SA-β-gal-positive cells in visfatin siRNA-transfected cells (Fig. 1C and D). We next investigated whether visfatin affected cellular senescence. Following exogenous visfatin treatment, the fraction of cells stained positive for SA-β-gal activity increased approximately 8-fold compared with that in the control (Fig. 1E and F); this effect was reversed by pretreatment with neutralizing anti-visfatin antibodies (Fig. 1E and F).

Toll-like receptor 4 blockade alleviated visfatin-induced cellular senescence

Recent studies have shown that visfatin directly binds to TLR4.[18,21] Thus, we examined the involvement of TLR4 in the induction of cellular senescence by visfatin in hDPCs. The basal level of TLR4 protein was observed in control hDPCs, and TRL4 protein was detected on the plasma membrane of hDPCs; however, the level was unchanged in visfatin-treated cells (Fig. 2A,B,E). Then, to elucidate the roles of TLR4 in visfatin-induced senescence, the hDPCs were pretreated with neutralizing anti-TLR4 antibodies prior to visfatin treatment. The increase in the number of SA-β-gal-positive cells elicited by visfatin was reversed by pretreatment with the neutralizing anti-TLR4 antibodies (Fig. 2C and D). Similar results were obtained using TAK-242, a specific chemical inhibitor of TLR4[23] (Figure s2A). The change in the fraction of SA-β-gal-positive cells among the visfatin-treated hDPCs was evaluated by detecting the expression of p21 and p53 proteins, which are well-known markers of senescence. Visfatin increased the protein levels of p21 and p53 in hDPCs (Fig. 2E, Figure s2A). When cells were pretreated with neutralizing anti-TLR4 antibodies prior to visfatin treatment, the visfatin-induced increases in p21 and p53 protein expression were significantly attenuated (Fig. 2E, Figure s2B,C).

Toll-like receptor 4 blockade attenuated visfatin-induced telomere damage

Cellular senescence is caused by DNA damage.[24] To examine the involvement of TLR4 in DNA damage response by visfatin in hDPCs, immunocytochemistry was performed to detect γH2AX foci formed at sites of damaged DNA and sites with uncapped dysfunctional telomeres. Visfatin

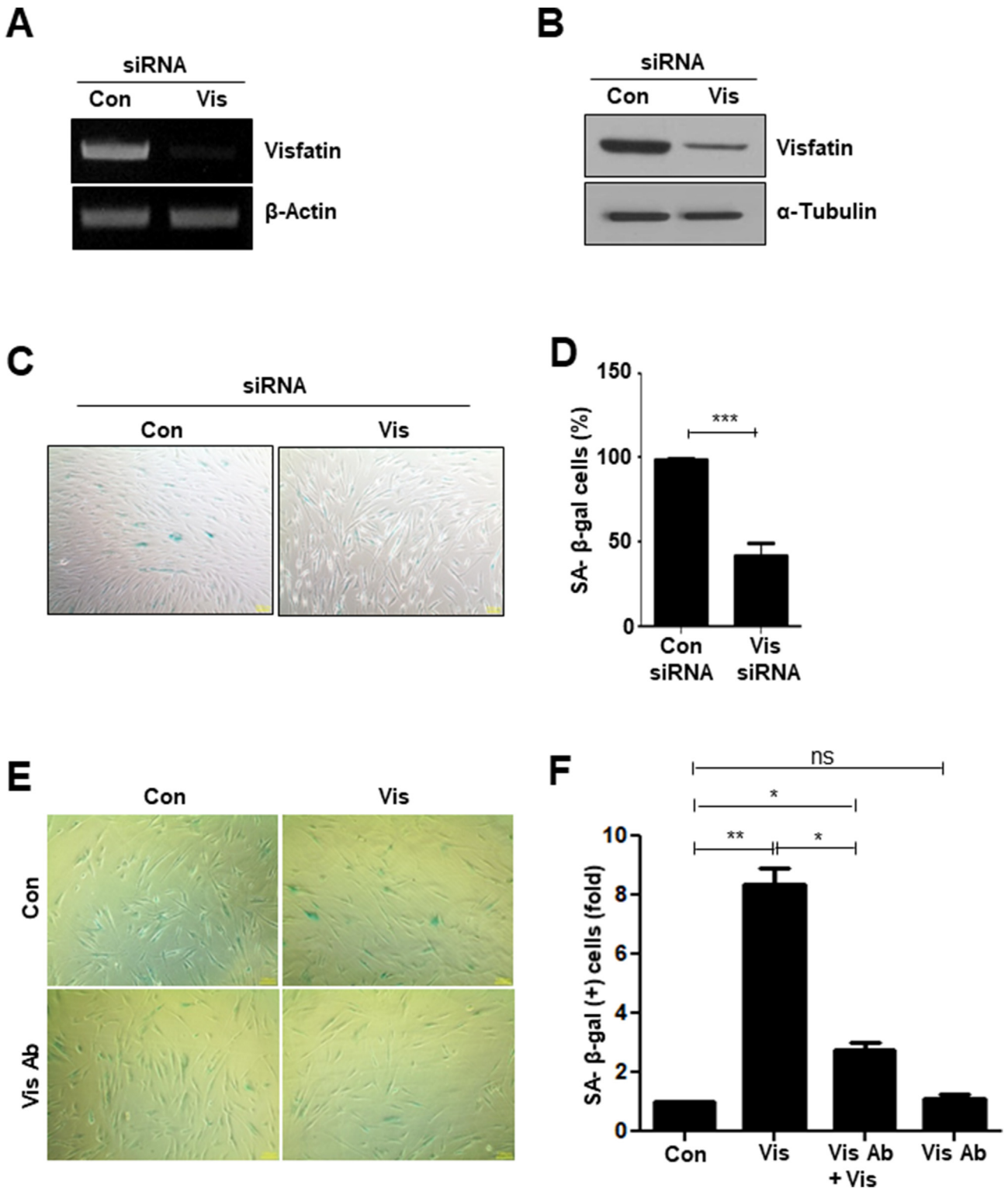

Fig. 1 Effects of visfatin on senescence in hDPCs. (A—D) hDPCs were transfected with control siRNA or visfatin siRNA for 48 h. (A) Total RNA was isolated and analyzed by RT-PCR to determine visfatin mRNA expression. β-Actin was used as an internal control. (B) Transfected cell lysates were subjected to western blotting for detection of visfatin levels; α-tubulin was used as the loading control. (C) Transfected cells were stained for detection of the activity of senescence-associated (SA)-β-galactosidase. Scale bar: 200 μm. (D) Quantification of the percentage of SA-β-galactosidase-positive cells. (E, F) hDPCs were pretreated with neutralizing anti-visfatin antibodies (10 μg/mL) for 3 h and then incubated with visfatin (500 ng/mL) for 24 h. (E) Cells were stained for detection of the activity of SA-β-galactosidase. Representative image of SA-β-galactosidase staining. Scale bar: 200 μm. (F) Quantification of the percentage of SA-β-galactosidase-positive cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

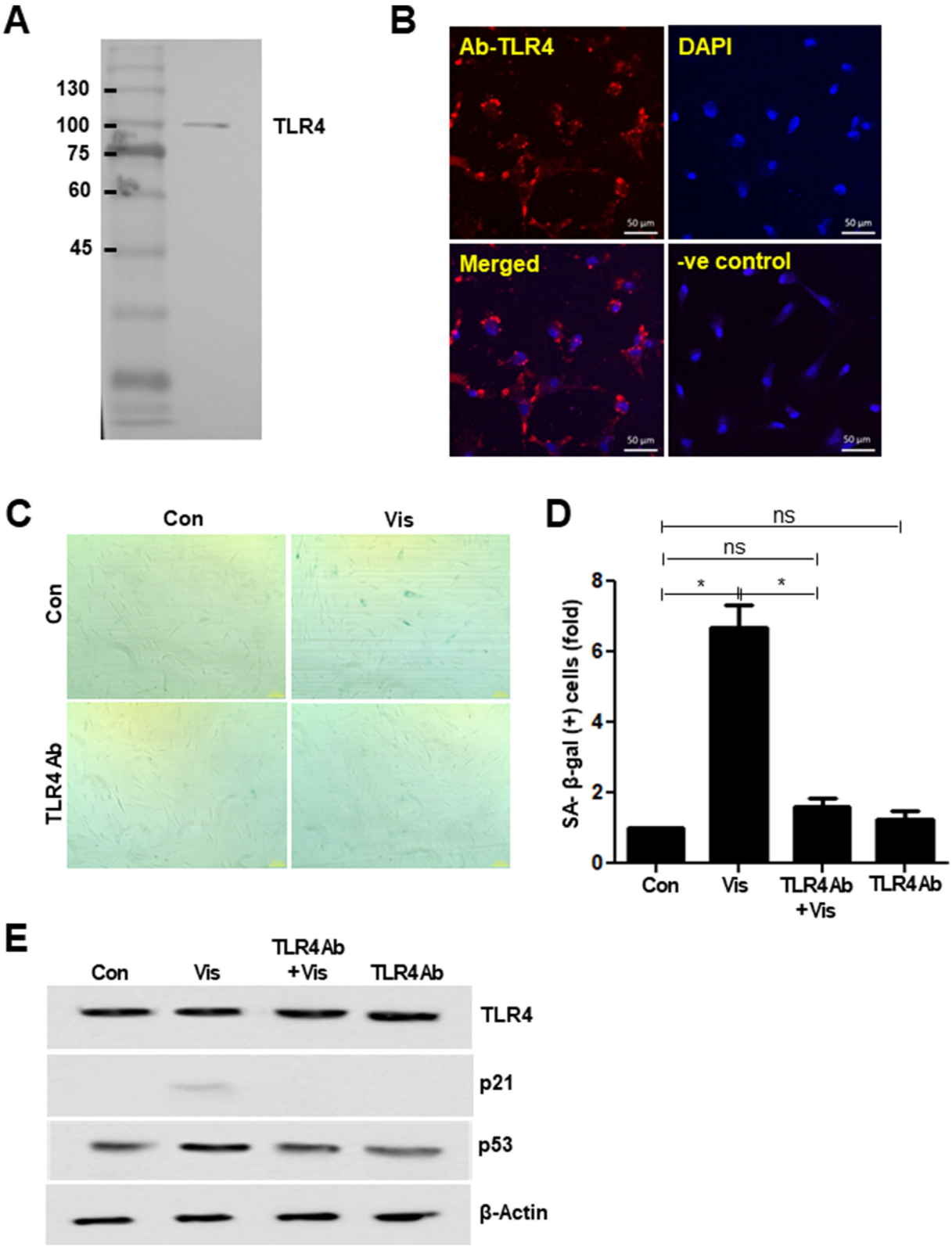

Fig. 2 Effects of TLR4 blockade on visfatin-induced senescence in hDPCs. (A) Cell lysates of hDPCs were subjected to western blotting for detection of the basal levels of TLR4 protein. (B) Cells were stained using anti-TLR4 antibodies (red). Nuclei were counterstained with DAPI (blue). Scale bar: 50 μm. (C—E) hDPCs were pretreated with neutralizing TLR4 antibodies (10 μg/mL) for 3 h and then incubated with visfatin (500 ng/mL) for 24 h. (C) Cells were stained for detection of the activity of SA-β-galactosidase. Representative image of SA-β-galactosidase staining. Scale bar: 200 μm. (D) Quantification of the percentage of SA-β-galactosidase-positive cells. (E) Cell lysates were subjected to western blotting for detection of visfatin levels; β-Actin was used as the loading control. *$P < 0.05$.

increased in the number and intensity of γH2AX signals (Fig. 3A–C). Moreover, visfatin caused an increase in the number of γH2AX foci that were colocalized with telomeric TRF-1 signals (Fig. 3A). Furthermore, the visfatin-induced increase in both the intensity and number of γH2AX-positive cells was significantly attenuated by pretreatment with neutralizing anti-TLR4 antibodies (Fig. 3A,C).

Toll-like receptor 4 blockade reversed visfatin-induced oxidative stress

Oxidative stress causes DNA damage, leading to premature senescence in cells.[25] Thus, we examined the involvement of TLR4 in visfatin-induced NADPH consumption and ROS production, two major indicators of oxidative stress.[25,26] Visfatin increased the $NADP^+$/NADPH ratio, which was reversed by treatment with neutralizing anti-TLR4 antibodies (Fig. 4A). Additionally, intracellular ROS levels were also markedly elevated after visfatin treatment in hDPCs, whereas neutralizing anti-TLR4 antibody pretreatment reversed the visfatin-induced increase in ROS levels (Fig. 4B and C). The stimulatory effects of visfatin on intracellular ROS production were verified using pyocyanin as a positive "ROS generator" control (Fig. 4B and C).

Toll-like receptor 4 blockade reduced visfatin-induced upregulation of inflammatory mediator-related gene expression

Activation of TLR4 promotes the production of various inflammatory mediators, including senescence-associated secretory phenotype (SASP) factors, such as IL-1β, IL-6, IL-8, COX-2, and TNF-α.[9,25] To investigate whether visfatin affected the expression of these genes, RT-PCR (Fig. 5A and B, Figure s3A–E) and qPCR (Fig. 5C–G) were performed. Visfatin increased the mRNA levels of *IL-1β*, *IL-8*, *IL-6*, *COX-2*, and *TNF-α*, and this effect was reversed by pretreatment with neutralizing anti-TLR4 antibodies in hDPCs (Fig. 5A–G).

Toll-like receptor 4 blockade inhibited visfatin-induced activation of mitogen-activated protein kinase and NF-κB signaling pathways

Because TLR4/mitogen-activated protein kinase (MAPK; e.g., ERK, JNK, and p38 MAPK) and TLR4/NF-κB signaling pathways are important regulators of inflammatory gene expression (including IL-1β, IL-6, IL-8, COX-2, and TNF-α),[9,12] Western blot analysis was performed to examine whether TLR4 is involved in the activation of MAPK and NF-κB signaling pathways during visfatin-induced senescence in hDPCs. Visfatin significantly elevated the phosphorylation of ERK1/2, JNK1/2, and p38 MAPK without affecting the levels of total ERK, JNK, and p38 (Fig. 6A, Figure s4A–C), and these increases were blocked by pretreatment with neutralizing anti-TLR4 antibodies. In addition, visfatin increased the levels of NF-κB p65 protein and the phosphorylation of serine 32 in IκBα (phospho-IκBα) but decreased the levels of IκBα protein; all of these effects were reversed by treatment with neutralizing anti-TLR4 antibodies (Fig. 6B, Figure s4D-F). Furthermore, visfatin-induced nuclear translocation of the p65 subunit of NF-κB was abrogated by treatment with neutralizing anti-TLR4 antibodies (Fig. 6C and D).

Discussion

This is the first study showing the involvement of TLR4 in visfatin-induced senescence of hDPCs. In this study, we found that TLR4 was expressed on hDPCs and that functional inhibition of TLR4 using neutralizing anti-TLR4 antibodies or TLR4 inhibitor prevented the induction of senescence in visfatin-stimulated hDPCs, suggesting that TLR4 may act as a key mediator of cellular senescence induced by visfatin.

Substantial progress has been made in understanding of the role of TLR4 in senescence/aging.[3,27–29] For example, the activation of TLR4 by LPS or S100A9 accelerates senescence of placental mesenchymal stem cells, human dental pulp stem cells, or bone marrow stromal cells.[3,27,28] TLR4 downregulation by PUM1 alleviates cellular senescence in human mesenchymal stem cells.[29] The role of TLR4 as a molecular initiator of cellular senescence is consistent with our results in hDPCs but inconsistent with the inhibitory effects of TLR4 on the senescence of other cell types, including skin fibroblasts and endothelial cells.[30,31] The reason for this discrepancy is unclear, but may be related to the cell type- and context-dependent effects of TLR4 signaling.

The aging phenotype is strongly associated with a state of systemic inflammation, called age-related inflammation (also referred to as inflammaging).[32] Inflammaging is a major risk factor of aging-related diseases.[32] Tooth aging is strongly related to pulp aging,[33,34] which gradually reduces pulp physiological activity through cellular senescence via accumulation of oxidative stress and production of SASP factors; these factors accompany age-related dental pulp inflammation, including pulpitis.[33-35] If left untreated, pulpitis leads to premature tooth loss and decreased quality of life.[1,2,35] Thus, discovering factors or signaling pathways that are commonly involved in senescence/aging and inflammation will be necessary for improving therapeutic approaches to the treatment of inflammaging-related diseases, including pulpitis. TLR4 is implicated in the pathogenesis of pulpitis,[36-38] and evidence indicates its important role in cellular senescence/aging.[29,39,40]. Although many studies have shown that visfatin-induced inflammatory responses mediate the TLR4/NF-κB pathway, no previous reports had shown whether TLR4 acts on visfatin-induced cellular senescence. Interestingly, our current results demonstrated that senescence induction by visfatin was regulated by TLR4, emphasizing the importance of the visfatin/TLR4 signaling axis in inflammaging.

Our results showed that visfatin was an endogenous TLR4 ligand, implying that visfatin could induce cellular senescence, at least in part, via TLR4 in hDPCs. At the same time, senescent hDPCs stimulated by visfatin may also secrete SASP factors through NF-κB and MAPKs and promote the development of inflammation, thereby exacerbating inflammaging in dental pulp tissue and contributing to pulpitis. Further investigations will improve our understanding of the mechanisms of pulpitis and facilitate the design of therapeutic interventions by targeting the visfatin/TLR4 signaling axis.

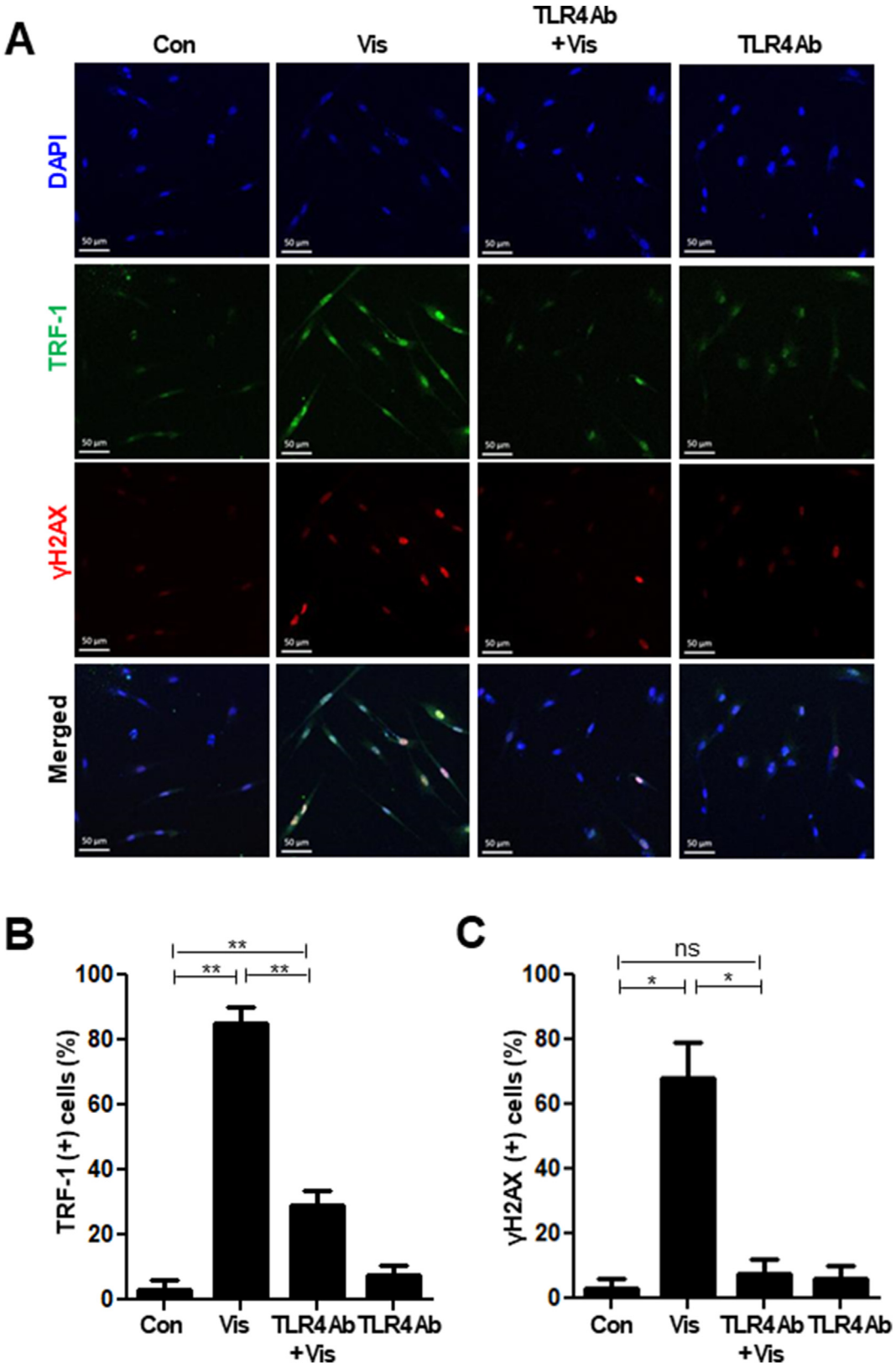

Fig. 3 Effects of TLR4 blockade on visfatin-induced telomere damage formation in hDPCs. hDPCs were pretreated with neutralizing anti-TLR4 antibodies (10 μg/mL) for 3 h and then incubated with visfatin (500 ng/mL) for 24 h. (A) Immunofluorescence analysis of TRF-1 (Alexa Fluor [AF]-488, green) and γH2AX (AF-594, red) in hDPCs. The cells were analyzed using a confocal microscope. Nuclei were counterstained with DAPI (blue). (B, C) Quantitative results for the percentages of TRF-1- and γH2AX-positive cells. $^{*}P < 0.05$, $^{**}P < 0.01$.

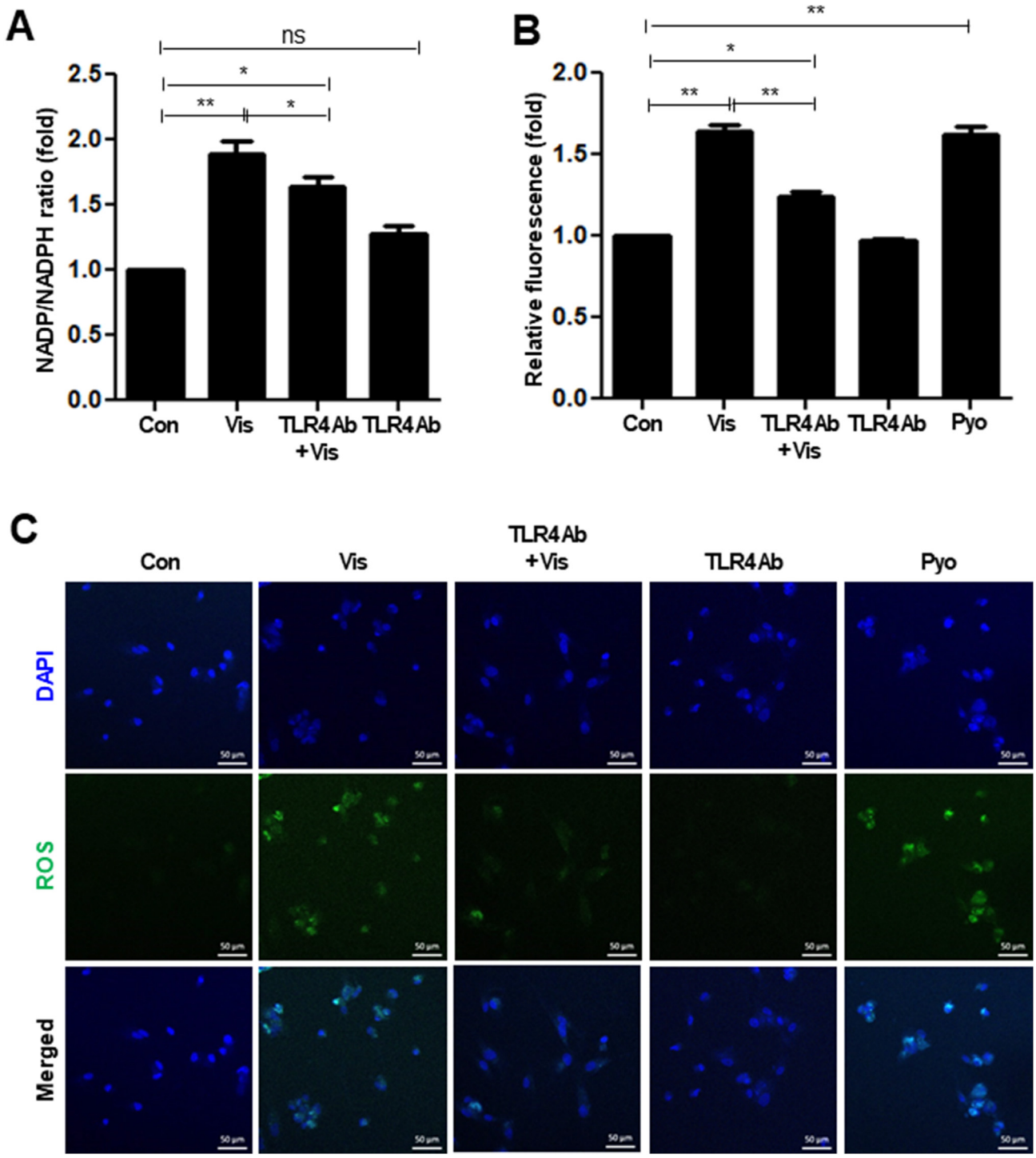

Fig. 4 Effects of TLR4 blockade on visfatin-mediated NADPH consumption and ROS generation in hDPCs. hDPCs were pretreated with neutralizing anti-TLR4 antibodies (10 μg/mL) for 3 h and then incubated with visfatin (500 ng/mL) for 24 h. (A) Measurement of the NADP(+)/NADPH ratio in visfatin-treated hDPCs with or without neutralizing anti-TLR4 antibody pretreatment. (B, C) Measurement of total intracellular ROS levels in visfatin-treated hDPCs with or without neutralizing anti-TLR4 antibody pretreatment using a fluorescent microplate reader (B) and confocal microscope (C). Pyocyanin was used as a positive control for ROS generation. $^{*}P < 0.05$, $^{**}P < 0.01$.

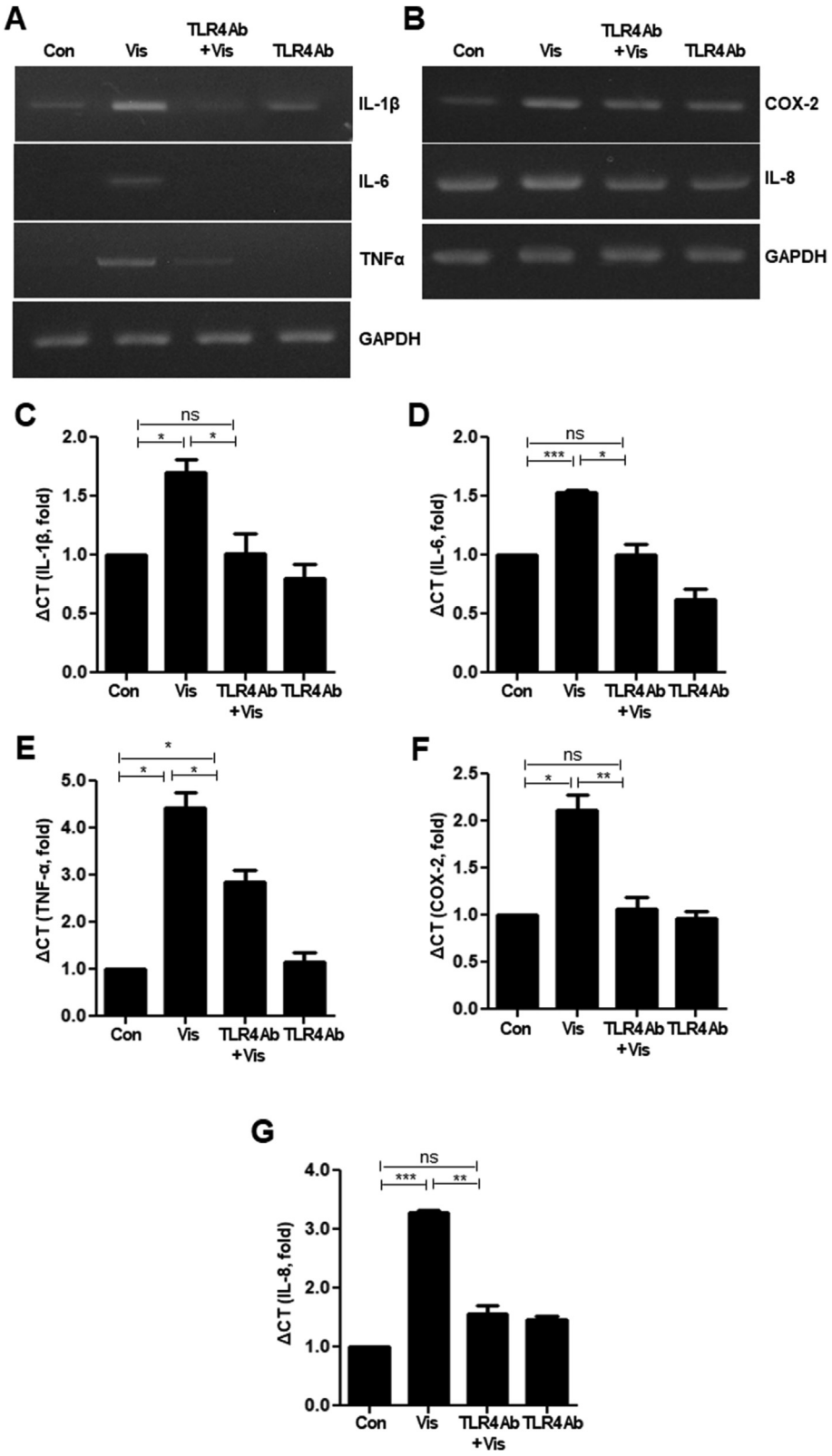

Fig. 5 Effects of TLR4 blockade on visfatin-induced expression of inflammatory SASP genes in hDPCs. hDPCs were pretreated with neutralizing anti-TLR4 antibodies (10 μg/mL) for 3 h and then incubated with visfatin (500 ng/mL) for 24 h. (A,B) Total RNA was isolated and analyzed by RT-PCR for determination of the mRNA levels of the indicated inflammatory mediators. *GAPDH* was used as an internal control. (C—G) Real-time RT-PCR was performed to determine the mRNA levels of inflammatory mediators. The expression level of the control (untreated) was set to 1, and the values were normalized to β-Actin mRNA levels. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

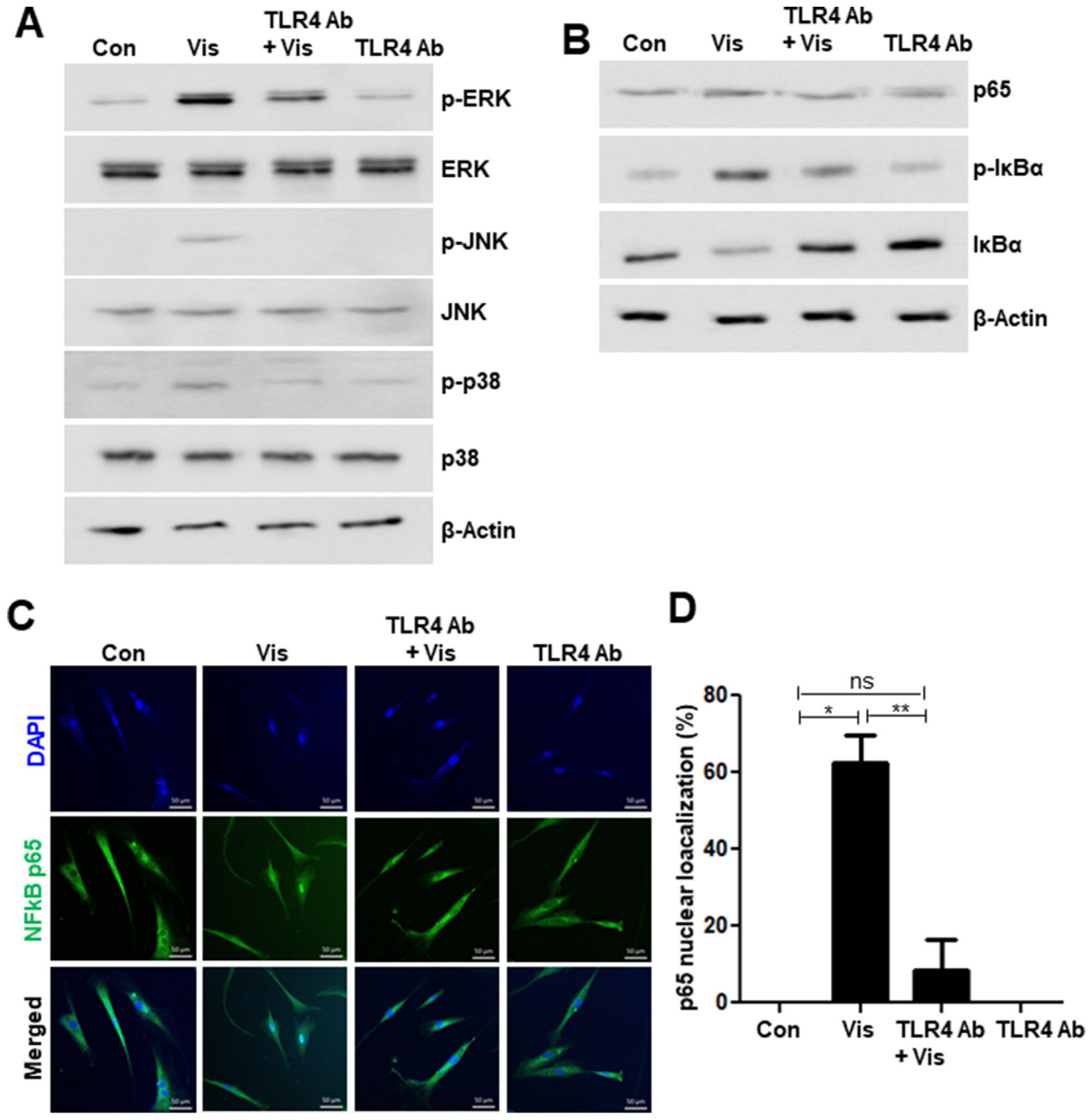

Fig. 6 Effects of TLR4 blockade on visfatin-induced activation of MAPK and NF-κB signaling pathways in hDPCs. hDPCs were pretreated with neutralizing anti-TLR4 antibodies (10 μg/mL) for 3 h and then incubated with visfatin (500 ng/mL) for 30 min. (A) Cell extracts were subjected to Western blot analysis for detection of phospho-ERK1/2, ERK1/2, phospho-JNK1/2, JNK1/2, phospho-p38 MAPK, and p38MAPK protein levels. β-Actin was used as the loading control. (B) The levels of NF-κB p65, phospho-IκBα, IκBα, and β-Actin proteins were determined by Western blot analysis. (C) Immunocytochemical analysis of NF-κB p65 protein localization in cells. The cells were visualized using a fluorescence microscope. Nuclei were counterstained with DAPI (blue). (D) Quantitative results for the percentage of nuclear localization of NF-κB p65. $^{*}P < 0.05$, $^{**}P < 0.01$.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2022.10.008.

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