Biomedicine & Pharmacotherapy 155 (2022) 113639

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Trimethylamine-N-oxide (TMAO) promotes balloon injury-induced neointimal hyperplasia via upregulating Beclin1 and impairing autophagic flux

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ARTICLE INFO

Keywords:
TMAO
Neointimal hyperplasia
Reendothelialization
Autophagic flux
Beclin1

ABSTRACT

Background and aims: TMAO is a microbiota-dependent metabolite associated with increased risk of various cardiovascular diseases. However, the relationship between TMAO and vascular injury-related neointimal hyperplasia is unclear. This study aimed to explore whether TMAO promotes neointimal hyperplasia after balloon injury and elucidate the underlying mechanism.

Methods and Results: Through hematoxylin and eosin staining and immunohistochemistry staining, we found that supplementary TMAO promoted balloon injury-induced neointimal hyperplasia, while reducing TMAO by antibiotic administration produced the opposite result. TMAO showed limited effect on rat aortic vascular smooth muscle cells (RAOSMCs) proliferation and migration. However, TMAO notably induced dysfunction of rat aortic vascular endothelial cells (RAOECs) in vitro and attenuated reendothelialization of carotid arteries after balloon injury in vivo. Autophagic flux was measured by fluorescent mRFP-GFP-LC3, transmission electron microscopy, and western blot. TMAO impaired autophagic flux, as evidenced by the accumulation of p62 and LC3II and high autophagosome to autolysosome ratios. Furthermore, we confirmed that Beclin1 level increased in TMAO-treated RAOECs and carotid arteries. Knocking down Beclin1 alleviated TMAO-induced autophagic flux impairment and neointimal hyperplasia.

Conclusions: TMAO promoted neointimal hyperplasia through Beclin1-induced autophagic flux blockage, suggesting that TMAO is a potential target for improvement of vascular remodeling after injury.

1. Introduction

Vascular remodeling commonly occurs in the pathological processes of vascular diseases, including atherosclerosis and hypertension, and vascular-related therapeutic interventions, such as angioplasty and endarterectomy. [1–4] Neointimal hyperplasia is the major pathological characteristic of vascular remodeling, which has been of much focus recently due to its associated adverse clinical events. [5,6] Vascular smooth muscle cells (VSMCs) and vascular endothelial cells (VECs) are important components of vessels. The prevailing theory suggests that excessive proliferation and migration of VSMCs and their metaplastic changes from the contractile phenotype to synthetic phenotype promote neointimal hyperplasia. [7–9] VECs, which primarily ensure vascular integrity, play an important role in neointimal hyperplasia. In situ

Abbreviations: TMAO, Trimethylamine N-oxide; RAOSMCs, rat aortic vascular smooth muscle cells; RAOECs, rat aortic vascular endothelial cells; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; TGF-β, transforming growth factor-beta; PCNA, proliferating cell nuclear antigen; CD31, platelet endothelial cell adhesion molecule-1.

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https://doi.org/10.1016/j.biopha.2022.113639
Received 20 July 2022; Received in revised form 25 August 2022; Accepted 30 August 2022
Available online 8 September 2022

endothelial cell repair relies on proliferation and migration of adjacent endothelial cells and endothelial progenitor cells. [10–12] VECs are not only nonpermeable barriers for protecting VSMCs against circulating growth-promoting factors but also modulators producing various cytokines involved in neointimal hyperplasia, such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor-beta (TGF-β). [3] Hence, maintenance of function and regeneration of VECs are essential for vascular homeostasis and prevention of neointimal hyperplasia. [13–15].

In the past decade, rapidly increasing evidence of the relationship between gut microbial organisms and cardiovascular diseases have accumulated. Trimethylamine N-oxide (TMAO) is a gut flora metabolite formed from trimethylamine (TMA); it is generated in digestive absorption of red meat, eggs, and salt-water fish that are rich in dietary choline and lecithin. Elevated plasma TMAO level is a predictor of major adverse cardiovascular events after adjustment for traditional risk factors in large independent clinical cohort studies. [16–18] Remarkably, TMAO plays a vital role in the development of hypertension, [19] vascular inflammation, [20,21] calcification, [22] and senescence. [23] However, the relationship between TMAO and vascular neointimal hyperplasia after balloon injury is largely unknown.

Autophagy, which is a conserved biological process regulating eukaryotic cell energy metabolism, growth, and death, is crucial for cellular homeostasis of cardiovascular diseases. [24–26] Previous studies have shown that the dysregulation of autophagic flux suppresses the viability of VECs [27,28] and induces vascular neointimal hyperplasia. [29,30] We recently exhibited that moderate autophagic flux inhibits neointimal hyperplasia. [31] Additionally, another study has shown that appropriate increase in autophagic flux in endothelial cell accelerates reendothelialization [32,33] and ameliorates intimal hyperplasia in rat carotid artery after balloon injury. [34,35] These demonstrate that increased autophagic flux is pivotal for VECs regeneration and vascular healing. Furthermore, TMAO has been proven to be involved in the regulation of autophagy. [36,37] However, whether TMAO is involved in the regulation of autophagic flux in neointimal hyperplasia remains unclear.

Beclin1 is well-recognized as one of the critical regulators of autophagic induction. [38] It mediates the localization of other autophagic proteins to pre-autophagosomal structures. Additionally, Beclin1 has been found to block autophagic flux, such as by impairing autophagosome clearance in myocardial ischemia and reperfusion injury. [39] Furthermore, excessive Beclin1 level inhibits proliferation of cancer cells and plays a crucial role in the regulation of inflammation, oxidative stress, and permeability of VECs. [40,41] However, the exact role of Beclin1 in autophagy-related neointimal hyperplasia remains unclear. Therefore, in this study, we aimed to define whether TMAO-blocked autophagic flux promoted neointimal hyperplasia through Beclin1 signaling pathway.

2. Materials and methods

2.1. Animal experiments

Male Sprague-Dawley (SD) rats aged 8–10 weeks, and weighted 200–250 g, were obtained from the Central Animal Care Facility of Southern Medical University. All animal care and experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals approved by the Institutional Animal Care Ethics Committee of the Southern Medical University (Guangzhou, China), and performed at Zhujiang Hospital. Regarding the carotid artery balloon injury model, rats were first anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal, i.p.). A 2 F Fogarty balloon catheter was inserted into the common carotid artery through the external carotid artery. The balloon was inflated and deflated with 0.2 mL of saline thrice to injure the vascular walls. Thereafter, 30ul Ad-shBeclin1 or Ad-shNC solutions (1 ×10^ 10 PFU/mL, WZ Biosciences Inc) were injected into the injured common carotid artery and incubated for 30 min, as previously described. [42] At 14 days after injury, all rats were sacrificed by pentobarbital overdose, and blood samples were collected from rat heart for analysis of TMAO levels. In the first in vivo study, rats were randomly divided into five groups (n = 6/group): (1) Sham group, (2) Injury group, (3) Injury + TMAO group, (4) Injury + Antibiotics (Abs) group, and (5) Injury + TMAO + Antibiotics (Abs) group. TMAO (150 mg/kg, 317594, Sigma-Aldrich, USA) or saline was injected intraperitoneally daily after injury until the end of the experiment. Antibiotics (vancomycin 100 mg/kg, neomycin 200 mg/kg, metronidazole 200 mg/kg, and ampicillin 200 mg/kg) [19,22] were administered daily to the vascular injury rat models by gavage. Thereafter, rats were divided into five groups (n = 5/group): (1) Sham group, (2) Injury group, (3) Injury + TMAO group, (4) Injury + TMAO + Ad-shNC group, and (5) Injury + TMAO + Ad-shBeclin1 group.

2.2. Hematoxylin and eosin (H&E) staining

Carotid artery sections were dyed using hematoxylin solution for 10 mins after deparaffinization and rehydration, and then stained with eosin dye solution for 30 s. Thereafter, the sections were sealed with neutral gum. The cross-sectional areas of the media and intima were measured using the image analysis software, Image Pro Plus 6.0. The intima/media (I/M) ratios were calculated.

2.3. Immunohistochemistry (IHC) staining

IHC analysis was performed, as previously described. [43] Rabbit polyclonal anti-PCNA (ab92552, Abcam) and anti-CD31 (BM0104, Boster) antibodies were used to evaluate neointimal thickness and reendothelialization rate, respectively. The tissue sections were observed by light microscopy.

2.4. Western blot analysis

Rat carotid arteries or cells were lysed using RIPA buffer to extract protein samples. The samples were then separated by SDS-PAGE and electro-transferred to polyvinylidene fluoride (PVDF) membranes. After blockage with 5% skimmed milk for 2 h, membranes were incubated with the primary antibodies overnight at 4 ℃. The primary antibodies included anti-cyclinD1 (ab16663, Abcam), anti-PCNA (ab92552, Abcam), anti-occludin (ab167161, Abcam), anti-p62 (ab109012, Abcam), anti-LC3B (ab192890, Abcam), anti-β-actin (ab8227, Abcam), and anti-Beclin1 (3738 S, CST). Subsequently, the membranes were incubated with secondary antibodies (BA1054, Boster) for 1 h at room temperature. The immuno-reactive bands were visualized with ECL kit (Engreen, Beijing, China), and the band intensities were determined using the Image Pro Plus 6.0 software.

2.5. Transmission electron microscopy (TEM)

The carotid artery tissues harvested from rats were immediately dipped in 2.5% glutaraldehyde at 4℃and left overnight. After tissue fixation, 2% aqueous uranyl acetate was added for 2 h. Before the tissues were sliced into small grids, they were dehydrated using graded alcohol. The grids were observed under Philips CM 10 electron microscope operated at 80 kV.

2.6. Cell culture and small interfering RNA (siRNA) transfection

Rat aortic vascular endothelial cells (RAOECs) were obtained from the BeNa Culture Collection (BNCC337856, Beijing, China). RAOECs were cultured in DMEM containing 10% FBS (Thermo Fisher Scientific, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in 5% CO_2 incubator. Rat aortic vascular smooth muscle cells (RAOSMCs) were isolated from thoracic aorta of rats, as described previously, [44] and cultured

under similar conditions. To determine the effects of TMAO on RAOECs and RAOSMCs, cells were grouped according to different concentrations of TMAO as follows (n = 3–6/group): 0 μM group, 100 μM group, 300 μM group, and 600 μM group. To investigate the effect of Beclin1 in TMAO-induced autophagic flux impairment, RAOECs were transfected with si-Beclin1 (50 nM) or si-NC (50 nM), and divided into four groups (n = 4 or 5/group): Control group, TMAO (300 μM) group, TMAO (300 μM) + si-NC group, and TMAO (300 μM) + si-Beclin1 group. si-Beclin1 (5′-GGACAAAGGCGCU CAAGUUTT-3′; 3′-AACUUGAGCGCCUUUGUCCTT-5′) and si-NC (SIID114558) were purchased from WZ Biosciences Inc.

2.7. Cell viability and proliferation assay

Cells were seeded in 96-well plates, and treated differently according to grouping. Post intervention, cell viability and proliferation were assessed using the CCK-8 assay kit (MCE, NJ, USA) and Edu assay kit (RiboBio, Guangzhou, China), according to the manufacturer's instructions.CCK-8 absorbance was measured at 450 nm using a microplate reader, and the EdU dyed images were captured by fluorescence microscopy. Image Pro Plus 6.0 19 software was used to count EdU-positive cells.

2.8. Cell migration assay

Cell scratch wound assay was performed in 6-well plates. At 90% confluency, cells were scratched with pipette tips and treated with starvation medium. The scratched areas were imaged immediately and 24 h later using a microscope, and the migration rates of cells were calculated using the Image Pro Plus 6.0. A transwell assay was used to assess cellular migration through a porous membrane, as described before. Briefly, cells (3×10^4 cells/well) were seeded in 24-well transwell plates (8 μm pore size, 6.4 mm diameter, VWR International) with media placed at the bottom of the transwell plates for 24 h. Thereafter, non-migrated cells on the upper surface of the transwell membrane were removed using a cotton bud, whereas migrated cells were stained with crystal violet (C0121, Beyotime, Shanghai, China), and counted by light microscopy.

2.9. Nitrite production assessment

Nitrite level was measured with kits (S0021S; Beyotime, Shanghai, China). Briefly, the RAOECs culture media were collected for measurement of nitrite concentration by the Griess reaction method. The absorbance of samples was measured at 540 nm using a microplate reader.

2.10. ROS level assessment

Intercellular ROS was detected using cell-permeable 2′,7′-dichlorofluorescein diacetate (DCFH-DA; KeyGen, Jiangsu, China). Briefly, cells were incubated with DCFH-DA and Hoechest 33258 for 30 mins in the dark at 37 ℃. Fluorescence intensity of DCFH-DA was recorded by fluorescence microscopy. To assess the oxidative stress level in vivo, freshly prepared frozen artery sections were incubated with 1 μmol/L fluorescent dye dihydroethidium (DHE, D7008, Sigma-Aldrich, USA) at 37 °C for 30 min in a humidified chamber and protected from light. Images were obtained with a fluorescence microscope.

2.11. Endothelial permeability measurement

RAOECs were cultured in 0.4 μm pore size 24-well transwell plates and treated, as indicated for 24 h. Subsequently, the bottom medium was replaced with 300 μl fresh media. A 100 μl fluoresceine isothiocyanate (FITC)–dextran (1 mg/mL, 10 kDa, Invitrogen) solution was added to the transwell insert, and the plate was incubated at 37 °C for 4 h to allow fluorescein molecules flow through the endothelial cell monolayer. The inserts were then removed, and fluorescent intensity in each well was determined at 488/516 nm using a fluorescent microplate reader. [45].

2.12. mRFP-GFP-LC3 puncta detection

Cells were seeded in confocal culture dishes. Adenovirus encoding tandem fluorescent mRFP-GFP-LC3 (MOI = 15) was used to transfect cells for 8 h. Before culturing under different conditions, cells were stabilized for 24 h in a normal medium. Subsequently, cells were fixed with 4% paraformaldehyde for 30 min and were stained with DAPI for 10 min. The images were obtained using a confocal fluorescence microscope. The number of autophagosomes (yellow dots) and autolysosomes (red dots) was calculated.

2.13. Statistical Analysis

Data are presented as mean ± SD. ONE-WAY ANOVA and Tukey post hoc test were used to test the differences among the groups. All statistical analyses were performed using Prism 9 (GraphPad Software, Inc). A p-value < 0.05 was considered statistically significant.

3. Results

3.1. TMAO promoted neointimal hyperplasia and inhibited reendothelialization after balloon injury

To demonstrate the role of TMAO in balloon injury-induced neointimal hyperplasia, rats were treated with exogenous TMAO or antibiotics, which could upregulate or downregulate plasma concentration of TMAO, respectively. Balloon injury produced a prominent ratio of intima/media thickness in the Injury group, as exhibited by H&E staining. Furthermore, a further increase in ratio of intima/media thickness was observed in the TMAO-treated injury group, whereas the ratio was decreased in the Injury + Abs group, compared with that in the Injury group. The injury group treated with TMAO and antibiotics had a lower ratio of intima/media thickness than the injury group treated with TMAO only and a higher ratio than the injury group treated with antibiotics only (Fig. 1A). As shown by IHC staining, PCNA positive cells, as indicators of neointimal hyperplasia, were remarkably increased or reduced in injured arteries after supplementary TMAO or antibiotic treatment, respectively. The Injury + TMAO + Abs group had more PCNA positive cells than those in the injury + Abs group, and less PCNA positive cells than those in the injury + TMAO group (Fig. 1B). Similarly, the results of the western blot were consistent with those of the IHC staining (Fig. 1C).

Reendothelialization plays a critical role in neointimal hyperplasia after vessel injury. [46] As shown in Fig. 1D, the rate of reendothelialization was monitored using the ratio of CD31 + cells cover length to lumen length of vessel. Compared with the Injury group, the Injury + TMAO group had an overtly reduced rate of reendothelialization. Contrary to the findings in the Injury + TMAO group, antibiotic treatment led to increased rate of reendothelialization. Moreover, The Injury + TMAO + Abs group displayed more CD31 + positive cells than those in the injury + TMAO group, and less CD31 + positive cells than those in the injury + Abs group.

These results showed that TMAO promoted neointimal hyperplasia and inhibited reendothelialization in balloon-injured arteries.

3.2. TMAO attenuated proliferation and migration of RAOECs

VECs and VSMCs are two major contributors involved in neointimal hyperplasia. [47] Our assessment investigating the effects of TMAO on RAOSMCs by CCK-8, EdU, scratch assay, and western blot showed that TMAO (100 μM, 300 μM, or 600 μM) did not significantly affect the proliferation, migration, and phenotypic switch of RAOSMCs

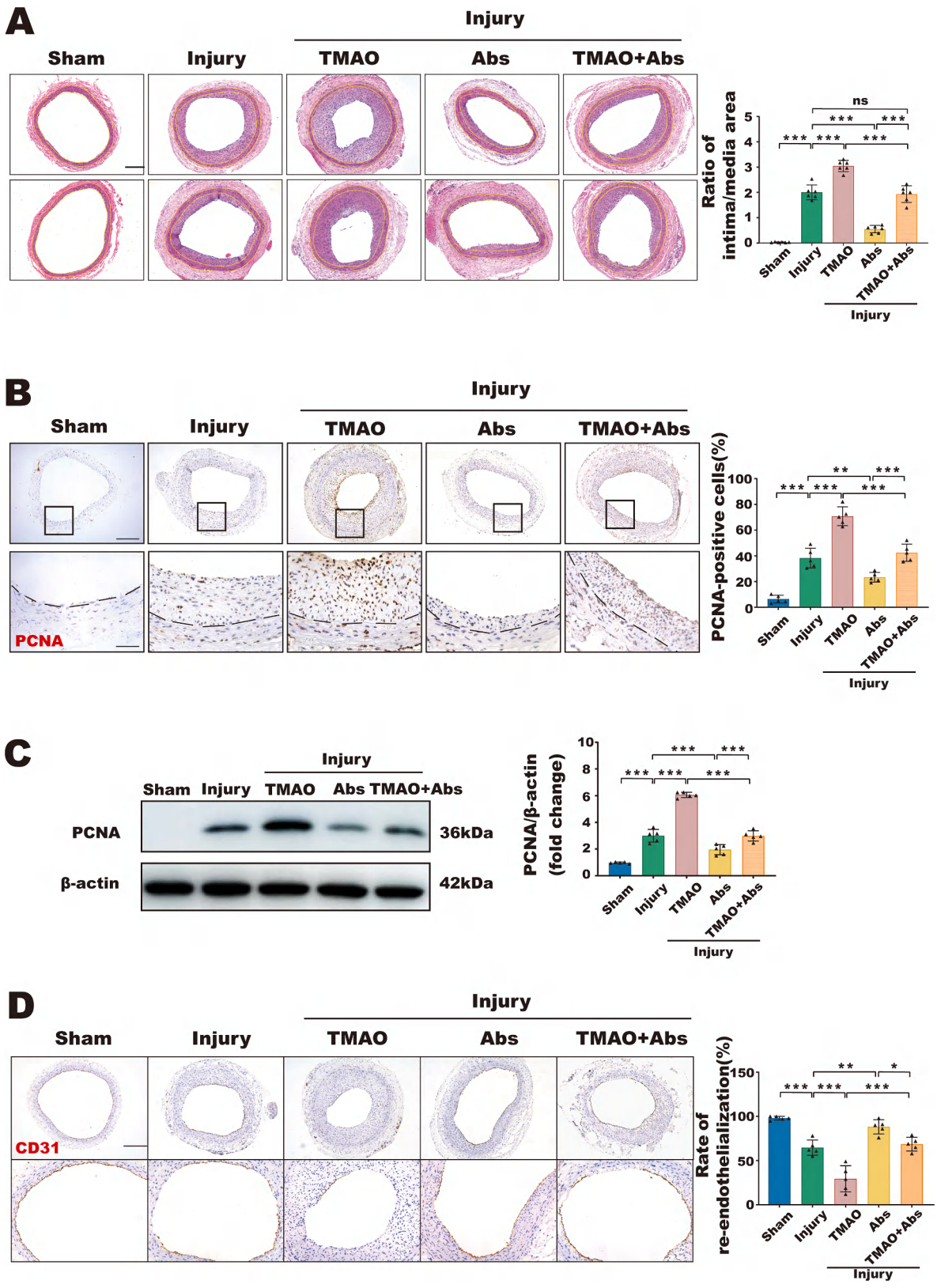

Fig. 1. TMAO promoted balloon injury-induced neointimal hyperplasia. A. Representative images and quantitative analysis of H&E staining of carotid arteries in different groups(n = 6). Scale bar: 200 μm or 50 μm. **B.** Representative images of immunohistochemistry staining of PCNA in the carotid arteries (n = 5). PCNA labeled nuclei were brown. Scale bar: 200 μm or 50 μm. **C.** The expression of PCNA in carotid arteries was detected by Western blot (n = 5). **D.** Representative images of immunohistochemistry staining of CD31 in the carotid arteries (n = 5). CD31 abeled nuclei were brown. Scale bar: 200 μm. Data represent the mean±SD; *P < 0.05, * *P < 0.01, * **P < 0.001.

(Fig. S1A-D in the Data Supplement), suggesting that the proliferation and migration of RAOSMCs were not regulated by TMAO directly in neointimal hyperplasia. Otherwise, we studied whether RAOECs were influenced by TMAO. The CCK-8 assay showed that TMAO inhibited the viability of RAOECs at doses of 300 μM and 600 μM (Fig. 2 A). Western blot showed that 300 μM and 600 μM of TMAO markedly decreased cyclin D1 (Fig. 2B), suggesting the effect of TMAO in inhibiting cell cycle progression of RAOECs. Similarly, high concentrations of TMAO resulted in decreasing percentage of EdU-positive cells (Fig. 2 C). These results revealed that TMAO inhibited the proliferation of RAOECs. Furthermore, the cell scratch assay and transwell assay were performed to detect the effect of TMAO on RAOEC migration. The data showed that the migration rates were markedly lowered by TMAO at doses of 300 μM and 600 μM, compared to that in the 0 μM group (Figs. 2D and 2E).

Taken together, these results demonstrated that TMAO suppressed RAOECs proliferation and migration.

3.3. TMAO induced RAOECs dysfunction

A previous study has indicated that endothelial dysfunction contributes to vascular remodeling after vessel injury. [48] Therefore, we intended to evaluate the effects of TMAO on endothelial function. The tight junctional architecture of endothelial cells, as a barrier in regulating paracellular permeability, maintains the integrity of the endothelium and vascular homeostasis. [49] Using in vitro studies, we tested whether TMAO affected the tight junctional architecture of RAOECs by assessing endothelial permeability and expression of the specific marker of tight junctions, occludin. As shown in Fig. 3A, 300 μM and 600 μM TMAO caused downregulation of occludin compared to that in the 0 μM group .300 μM and 600 μM TMAO also significantly increased endothelial permeability compared to that in the 0 μM group **(**Fig. 3B). These findings suggest that TMAO destroyed the tight junctional architecture of RAOECs. NO production in endothelial cells has a positive effect on vasodilation. It can inhibit smooth muscle cell proliferation and platelet activation. [48,50] To assess the effect of TMAO on NO production, we quantified nitrite level in RAOECs and found that 300 μM and 600 μM TMAO overtly reduced the nitrite level (Fig. 3C). ROS level, an oxidative stress indicator related to endothelial function, was detected. Obviously, TMAO increased ROS level in RAOECs by a concentration-dependent manner (Fig. 3D). 300 μM TMAO had a significant effect on cell proliferation and function, while 600 μM TMAO did not distinctly increase this effect. We chose 300 μM for follow-up in vitro experiments, in conformity with previous studies reported. [19,22] Furthermore, the DHE staining was performed in vessel tissue, the result showed TMAO induced excessive ROS level in injured vessel, but Abs reduced ROS production in injured vessel (Fig. 3E).

3.4. TMAO promoted neointimal hyperplasia through Beclin1-induced autophagic flux blockage

Considering that autophagic flux has been shown to participate in neointimal hyperplasia after balloon injury, [31,34,51] we explored whether TMAO aggravated neointimal hyperplasia by impairing autophagic flux. Therefore, p62, Beclin1, and LC3II, which were the specific markers of autophagy, were detected by western blot. Compared with the Sham group, the group treated with TMAO had distinctly upregulated p62, Beclin1, and LC3II levels at 14 days after vascular injury (Fig. 4A). Subsequently, we observed that TMAO generated noticeable accumulation of autophagosome through transmission electron microscopy in the balloon-injured vessel, revealing that TMAO impaired autophagosome clearance (Fig. 4B). These findings corroborated the effect of TMAO on blocking autophagic flux in balloon-injured carotid arteries.

As Beclin1 is a classical regulator of autophagy, we assessed the role of Beclin1 in TMAO-induced neointimal hyperplasia in vivo, and transfected adenovirus to knock down Beclin1 in carotid arteries and confirmed the transfection efficiency (Fig. 5A). Thereafter, we investigated the autophagy level after Beclin1 knockdown. The western blot results showed that TMAO-induced over-expression of p62 and LC3II was ameliorated after downregulation of Beclin1 at 14 days after balloon injury. Meanwhile, the western blot of PCNA showed that the level of PCNA was significantly enhanced by TMAO in the balloon-injured vessels. However, this upregulation was reversed after silencing of Beclin1 (Fig. 5A). Consistently, H&E staining showed that I/M ratio was decreased in the Injury + TMAO + Ad-shBeclin1 group, compared to that in the Injury + TMAO + Ad-shNC group (Fig. 5B). As shown by CD31 stanning in Fig. 5C, the Injury + TMAO group had an overtly reduced rate of reendothelialization compared to that in the Injury group, but Beclin1 knockdown could efficiently remedy this situation.

All together, these data indicated that the Beclin1-mediated autophagic flux impairment was essential for neointimal hyperplasia in the TMAO-treated vessel.

3.5. TMAO-impaired autophagic flux promoted RAOECs dysregulation via activating Beclin1

To further determine whether TMAO impaired autophagic flux in RAOECs, we performed a western blot and observed that p62, Beclin1, and LC3 II were significantly upregulated in the TMAO groups at different concentrations (Fig. 6A). Furthermore, autophagic flux was monitored using the tandem mRFP-GFP-LC3 adenovirus in each group (Fig. 6B). Compared with the 0 μM group, the 300 μM and 600 μM TMAO groups had a markedly increased ratio of autophagosomes to autolysosomes, suggesting that TMAO blocks conversion of autophagosomes into autophagolysosomes and results in impairment of autophagic flux in RAOECs.

Beclin1 as an autophagy regulator, has been proven to not only regulate autophagosome formation but also affect the fusion of autophagosomes and lysosomes. [39] To further clarify the role of Beclin1 in regulating autophagic flux of RAOECs, Beclin1 was knocked down by siRNA. Successful downregulation of Beclin1 was confirmed by western blot (Fig. 7A). After silencing Beclin1, 300 μM TMAO-induced autophagic flux inhibition was partly recovered. The recovery was corroborated by western blot and mRFP-GFP-LC3 puncta detection, which assessed the reversion of p62 and LC3II expression and autophagosome to autolysosome ratio respectively (Figs. 7A and 7B). TMAO-induced reduced expression of cyclinD1 was altered after knocking down Beclin1 (Fig. 8A). Moreover, the EdU-positive cells were notably reduced in the TMAO group, compared with the control group, but this reduction was overtly reversed after deletion of Beclin1 (Fig. 8B). The cell scratch revealed a similar result as that of the EdU assay, the adverse effect of TMAO on RAOEC migration was improved in the TMAO + si-Beclin1 group, compared with that in the TMAO + si-NC group (Fig. 8C). Collectively, these data manifested that TMAO promoted Beclin1 expression, which impaired autophagic flux and inhibited proliferation and migration of RAOECs.Fig. 9.

4. Discussion

In this study, we elaborated that TMAO promoted neointimal hyperplasia in vivo and inhibited proliferation and migration of ECs in vitro. Specifically, we further identified that TMAO induced upregulation of Beclin1, which subsequently impaired autophagic flux. Impaired autophagic flux was responsible for these effects of TMAO. To our knowledge, this study was the first to show that TMAO promotes vascular neointimal hyperplasia through Beclin1-mediated impairment of autophagic flux.

Accumulating evidence shows an important association between gut microbe-derived metabolites and development of cardiovascular diseases. [16,52–54] Trimethylamine N-oxide (TMAO), as one of these metabolites, has been got noticed due to the associated potential risk of

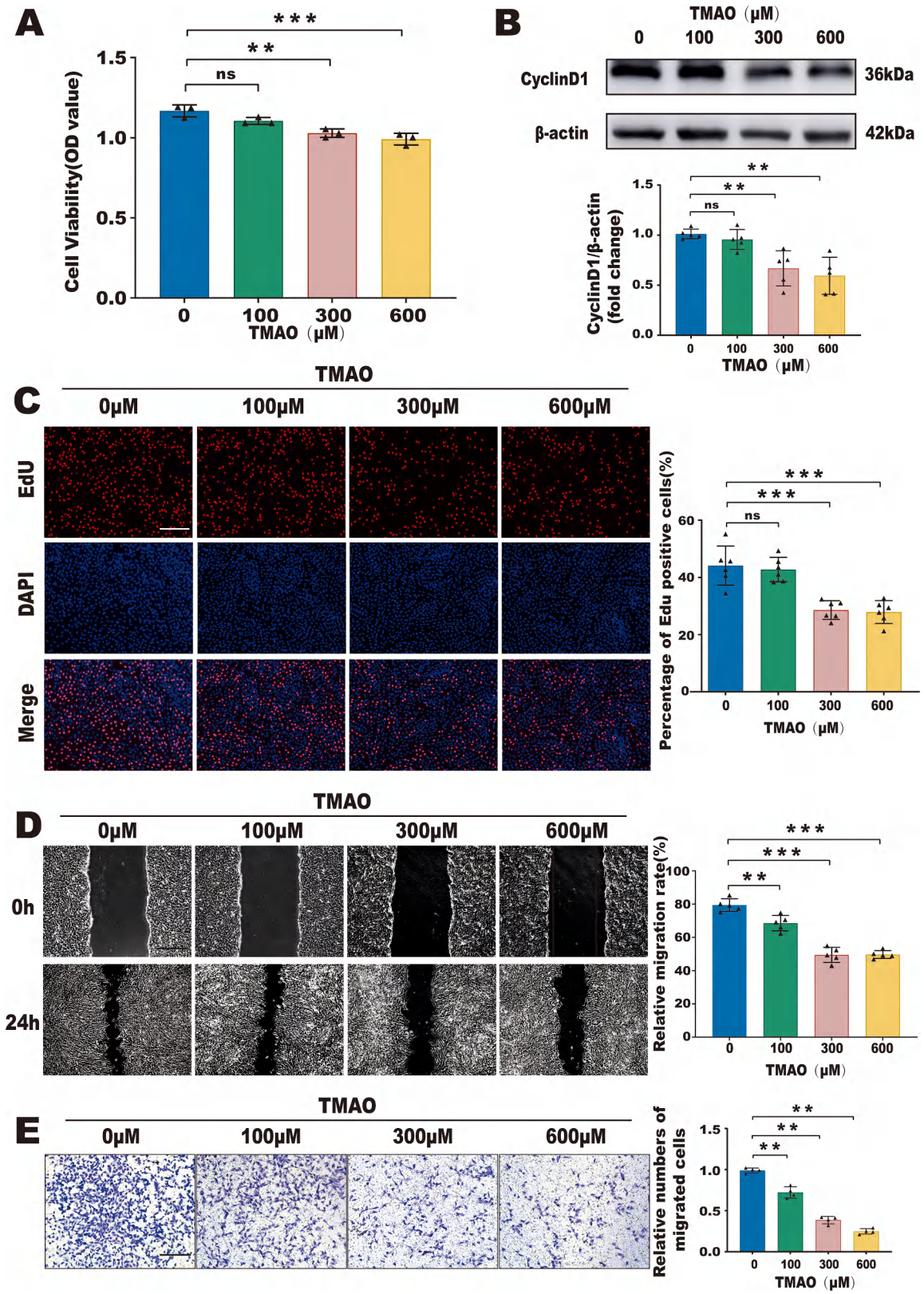

Fig. 2. TMAO inhibited proliferation and migration of RAOECs. A. The cell viability of RAOECs was assessed using CCK-8 assay. **B.** The expression of CyclinD1 in TMAO-treated RAOECs was detected by western blot. **C.** RAOECs proliferation was detected by EdU assay. Representative images and quantitative analysis of EdU-positive cells. The red dots represented the proliferating cell. The blue dot represented DAPI. Scale bar: 250 μm. **D&E.** The migration of RAOECs was measured by scratch assay and transwell assay. The representative images of migrated cells at 0 h and 24 h after scratch. Scale bar: 500 μm. Data represent the mean±SD; * *P < 0.01; * **P < 0.001; ns indicates not significant.

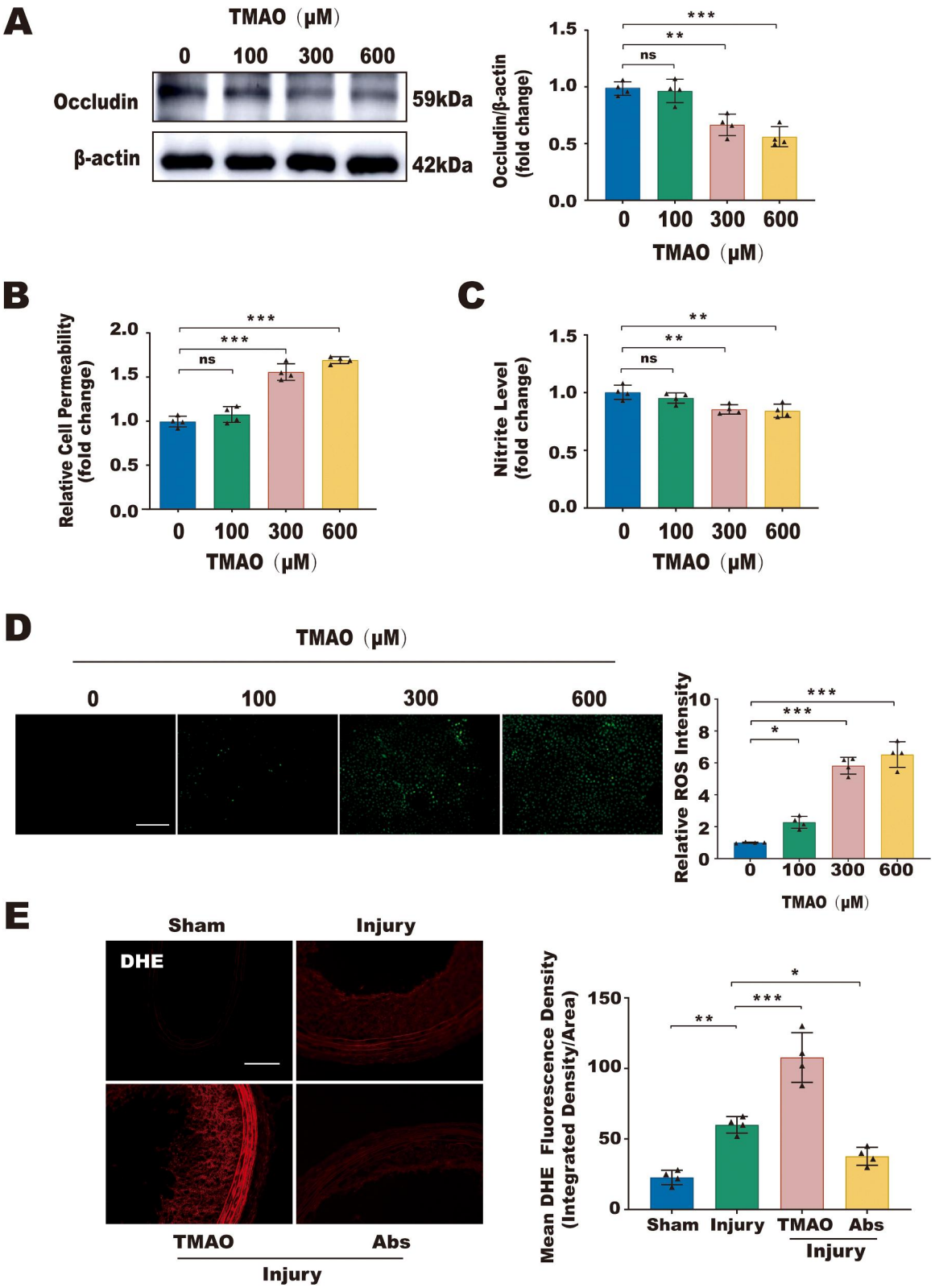

Fig. 3. TMAO induced RAOECs dysfunction. A. The expression of occludin in RAOECs was detected by western blot. **B.** Cell permeability was measured by FITC–dextran (10 kDa). **C.** Nitrite production in the RAOECs was detected using the Griess kit (n = 4). **D.** The intracellular ROS level of RAOECs was verified by DCFH-DA. The Scale bar: 250 μm. **E.** Representative images of vascular DHE staining (n = 4). The Scale bar: 50 μm Data represent the mean±SD; * *P < 0.01; * **P < 0.001; ns indicates not significant.

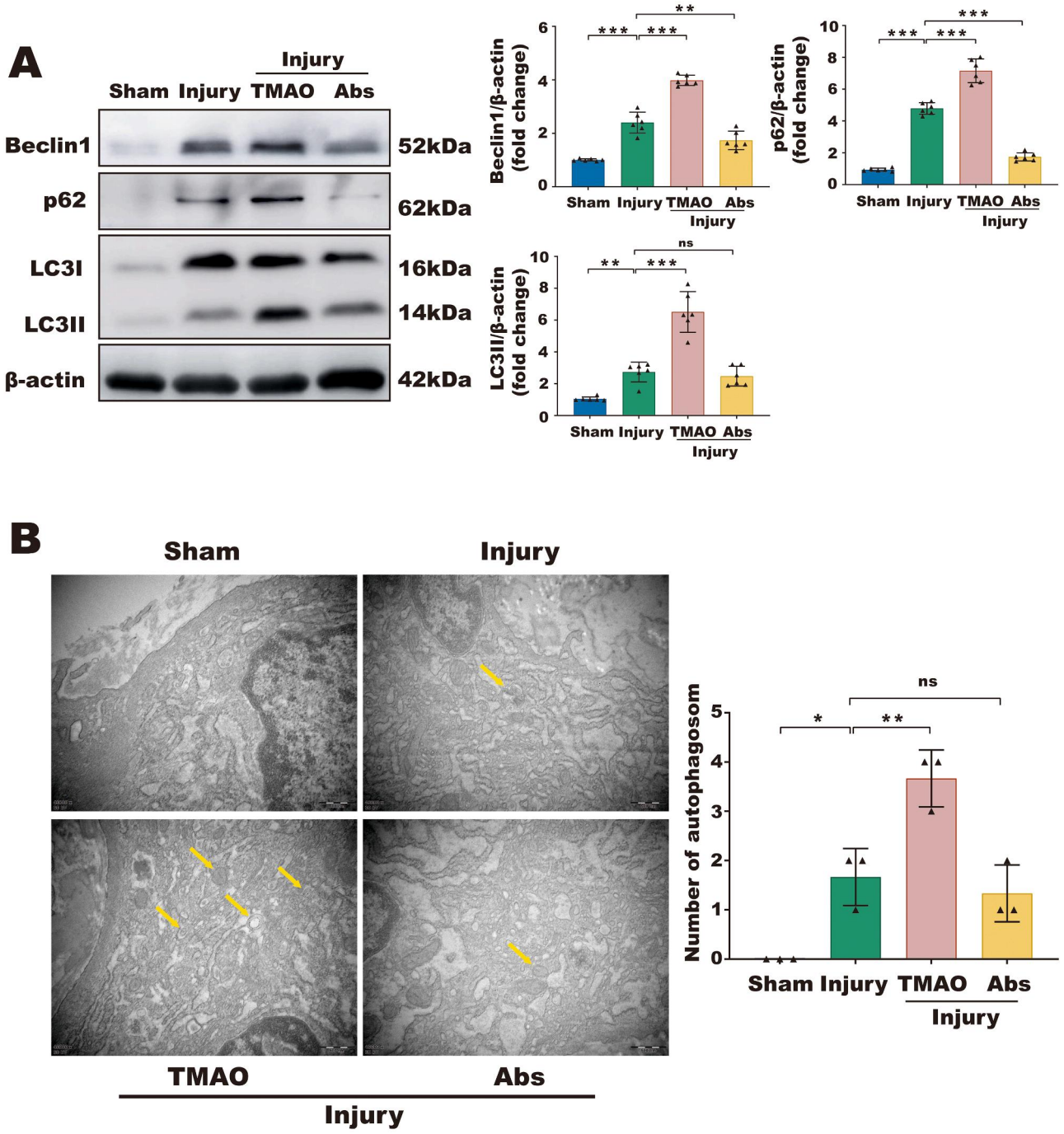

Fig. 4. TMAO impaired autophagic flux in balloon-injured carotid arteries. A. The protein expression level of Beclin1, p62, and LC3 II in the carotid arteries was detected by western blot (n = 6). **B.** Representative images of autophagosomes in rat carotid arteries obtained by transmission electron microscopy in different groups (n = 3). Autophagosomes were labeled with yellow arrows. Scale bar: 500 nm. Data represent the mean±SD; *P < 0.05; * *P < 0.01; * **P < 0.001; ns indicates not significant.

atherosclerosis, [55,56] hypertension, [19] vascular aging, [23] heart failure, [57,58] and stroke. [59] Elevated plasma TMAO is associated with a higher risk of future major cardiac events in patients undergoing coronary angiography. [60–62] Particularly, vascular remodeling, which is characterized by neointimal hyperplasia, plays a pivotal role in the development of cardiovascular diseases. In our study, we found that elevated plasma TMAO promoted balloon injury-induced artery neointimal hyperplasia in rats. Our study corroborated the findings of previous studies, indicating TMAO is a risk factor for cardiovascular diseases.

Considering that VSMCs pathological change, including phenotypic switching from contractile phenotype to Secretory phenotype and abnormal media-to-intima migration and proliferation, [7–9] are important cellular processes during neointimal hyperplasia. We have examined the effect of TMAO on RAOSMCs. However, no obvious effects of TMAO (0–600 μM) on the proliferation, migration, and phenotypic switching of RAOSMCs were found, suggesting that TMAO's effect on neointimal hyperplasia is not by a direct regulation of RAOSMCs. Notably, TMAO inhibited reendothelialization in injured vessels. ECs are considered as primarily responsible for maintenance of vascular homeostasis. Once ECs are damaged, multiple pathophysiological changes, such as thrombus formation, inflammation, and smooth muscle cell

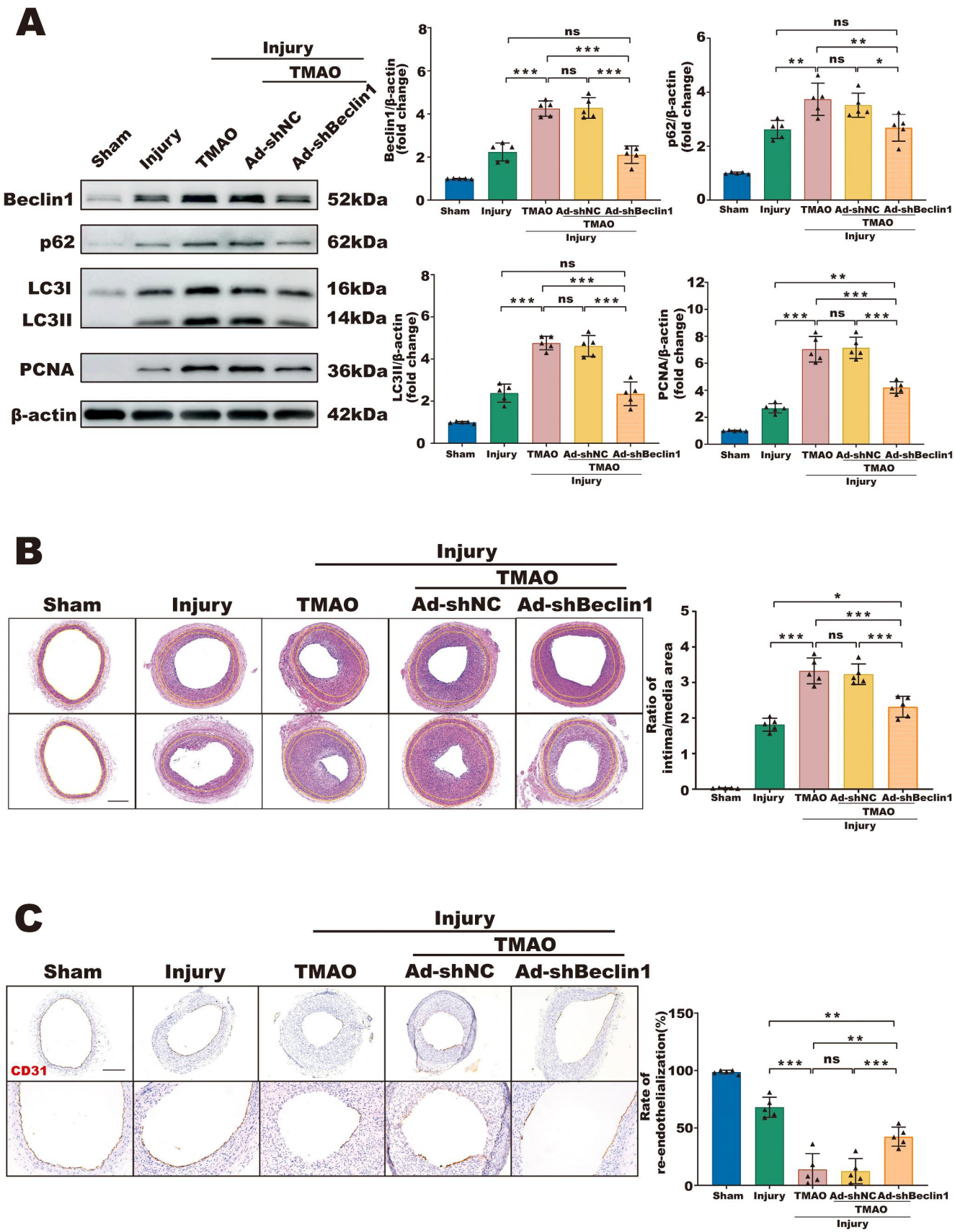

Fig. 5. Beclin1 knock-down restored autophagic flux and attenuated TMAO-induced neointimal hyperplasia. A. The protein expression of Beclin1, p62, LC3II, and PCNA in carotid arteries were determined by western blot (n = 5). **B.** Representative images and quantitative analysis of H&E staining of carotid arteries in different groups (n = 5). Scale bar: 200 μm. **C.** Representative images of immunohistochemistry staining of CD31 in the carotid arteries (n = 5). CD31 labeled nuclei are brown. Scale bar: 200 μm. Data represent the mean±SD; *P < 0.05; * *P < 0.01; * **P < 0.001; ns indicates not significant.

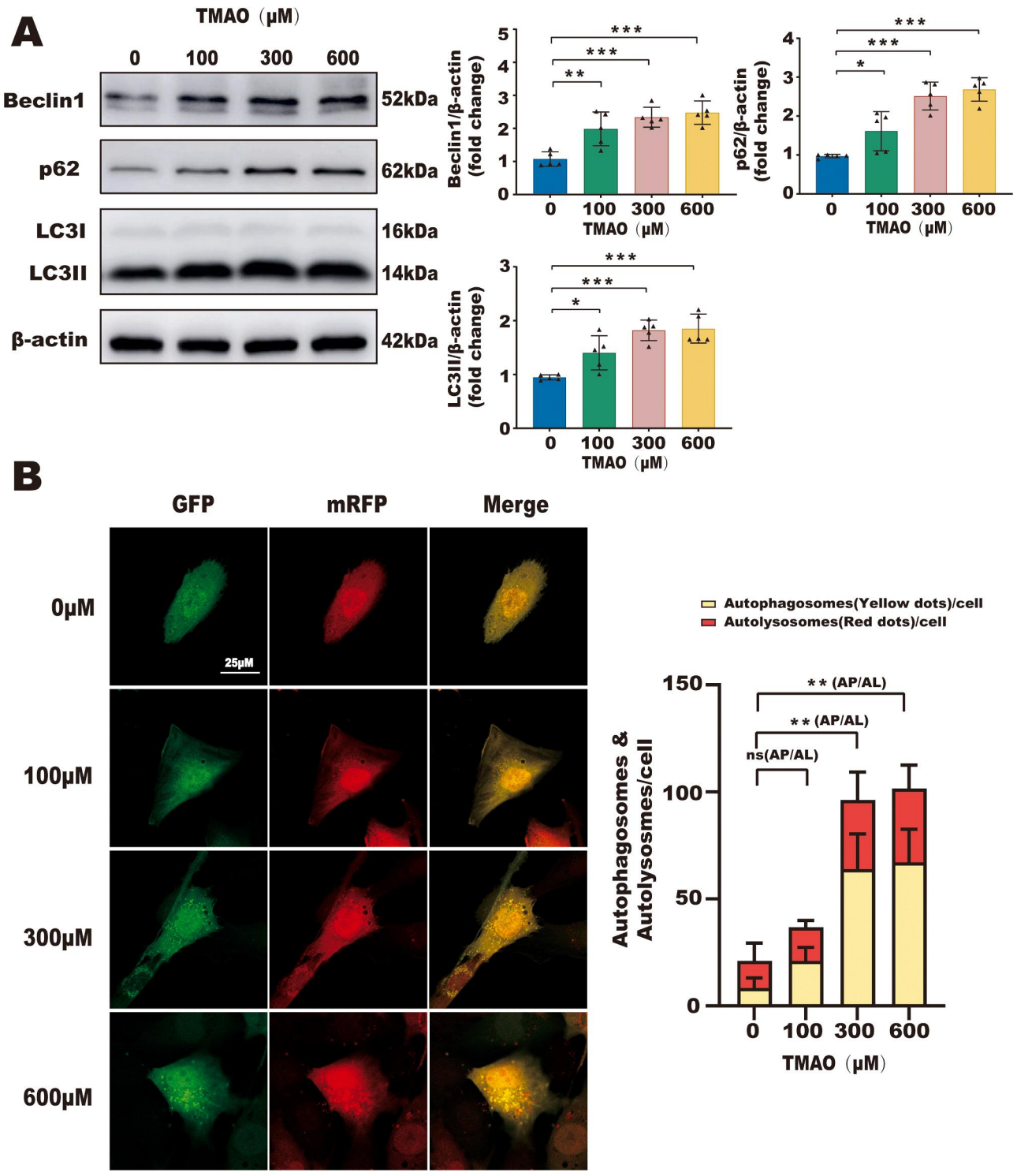

Fig. 6. TMAO impaired autophagic flux in TMAO-treated RAOECs. A. The protein expression level of Beclin1, p62, and LC3 II in RAOECs was detected by western blot. **B.** RAOECs transfected with adenovirus harboring tandem fluorescent mRFP-GFP-LC3 for monitoring autophagic flux. Representative images of immunofluorescent RAOECs were shown. GFP dots are green, and mRFP dots are red. Scale bar represents 25 μm. Data represent the mean ± SD; *P < 0.05; * *P < 0.01; * **P < 0.001; ns indicates not significant.

hyperproliferation, occur and ultimately lead to neointimal hyperplasia. [63–65] According to previous studies, repair of damaged ECs and promotion of reendothelialization are crucial for vascular healing. [66, 67] In our study, TMAO directly inhibited proliferation and migration of ECs. In vivo, we speculated that TMAO-induced similar effects on ECs and finally contributed to subendothelial tissue exposure, which could accelerate neointimal hyperplasia. Consistent with previous studies, our study found that TMAO caused EC dysfunction, including increased paracellular permeability, promoted oxidative stress, and decreased production of NO. [21,68,69] Additionally, DHE staining of arterial tissues suggested that TMAO promotes oxidative stress in vivo. Contrary to other studies, our study demonstrated the distinct responses of RAOECs and RAOSMCs to TMAO in vascular remodeling through assessing proliferation and migration. Moreover, TMAO inhibited NO

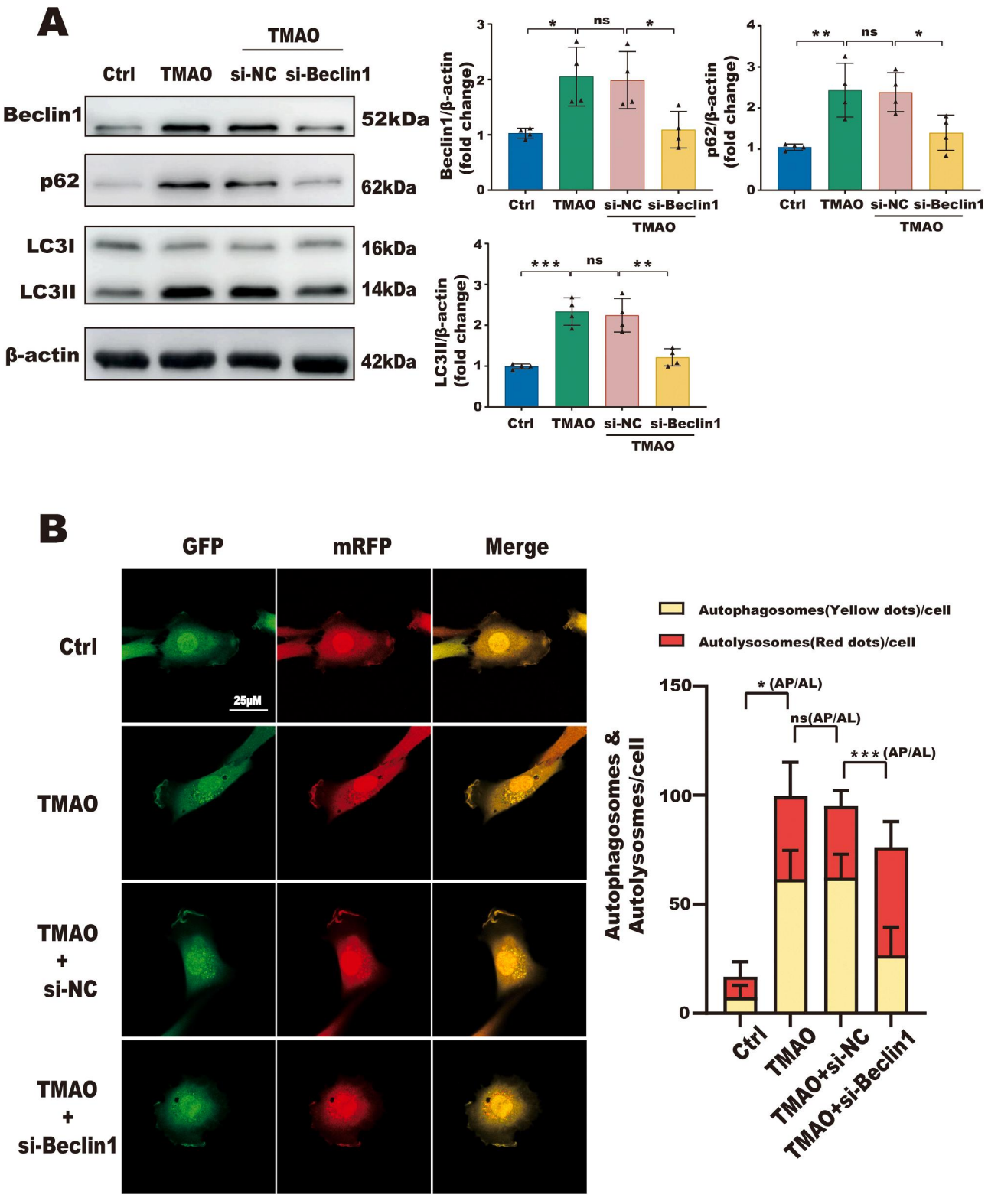

Fig. 7. Silencing of Beclin1 restored autophagic flux in TMAO-treated RAOECs. A. The protein expression levels of Beclin1, p62, and LC3 II in RAOECs were detected by western blot. **B.** The autophagic flux of RAOECs was monitored by adenovirus harboring tandem fluorescent mRFP-GFP-LC3. Representative images of immunofluorescent RAOECs are shown. GFP dots are green, and mRFP dots are red. Scale bar represents 25 μm. Data represent the mean±SD; *P < 0.05; * *P < 0.01; * **P < 0.001; ns indicates not significant.

production, which causes vasodilation and suppression of platelet aggregation. These findings revealed that TMAO might be a potential contributor in some cardiovascular diseases, such as coronary heart disease and vascular thrombosis.

Autophagy, an important physiological activity for maintaining the normal function of eukaryotic cells, is an intracellular lysosomal degradative process operative in homeostatic clearance of organelles and protein aggregates. [70] Our previous study indicated that

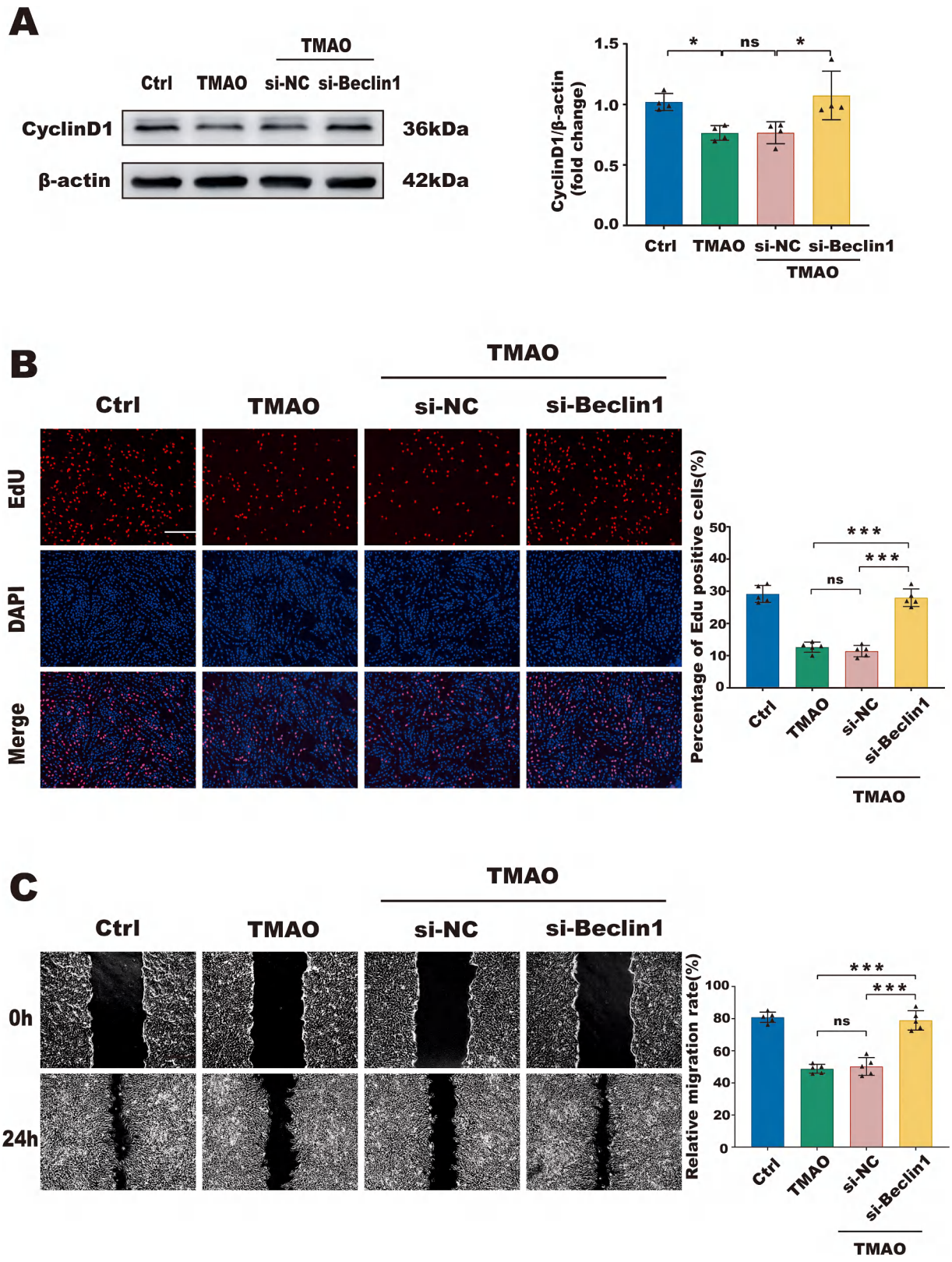

Fig. 8. Silencing of Beclin1 improved proliferation and migration in TMAO-treated RAOECs. A. The protein expression levels of CyclinD1 in RAOECs were detected by western blot. **B.** RAOECs proliferation was detected by EdU assay. Representative images and quantitative analysis of EdU-positive cells. The red dots represent the proliferating cell. The blue dot represented DAPI. Scale bar: 250 μm. **C.** The migration of RAOECs was measured using the scratch assay. The representative images of migrated cells at 0 h and 24 h after scratch. Scale bar: 500 μm. Data represent the mean±SD; *P < 0.05; * **P < 0.001; ns indicates not significant.

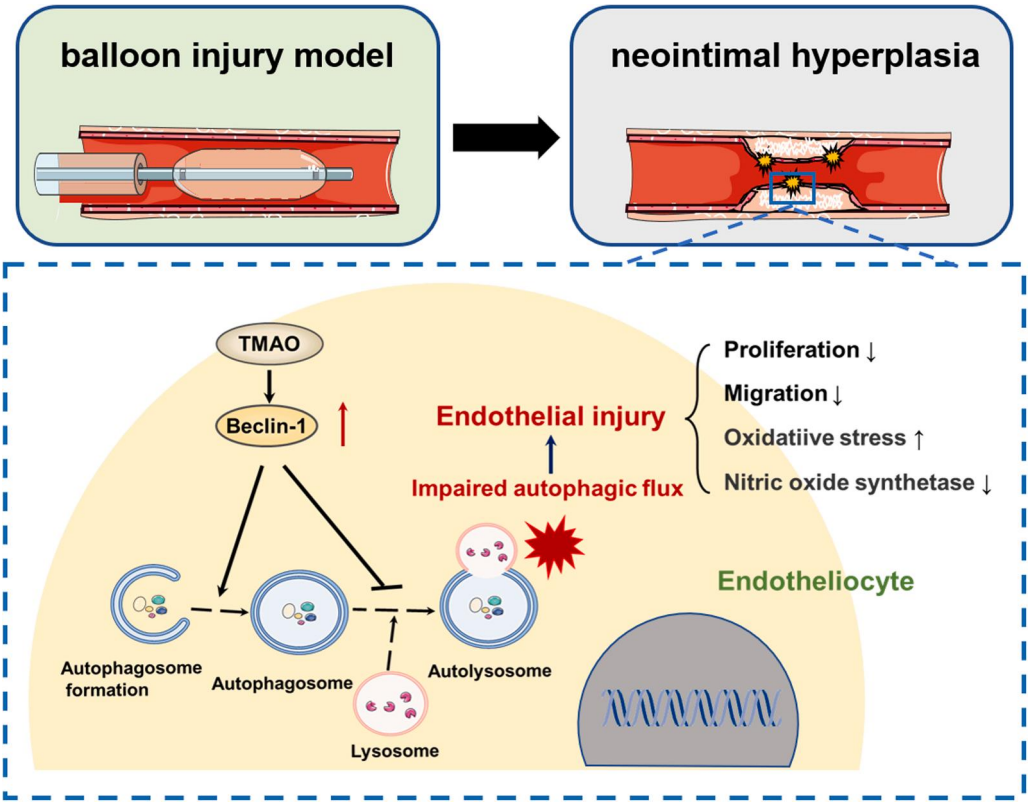

Fig. 9. TMAO aggravates balloon injury-induced neointimal hyperplasia via upregulating beclin1 and impairing autophagic flux.

moderate autophagy, which represented the unobstructed autophagic flux, inhibited vascular neointimal hyperplasia. [31] However, other studies showed that the abnormal autophagy is closely relevant to neointimal hyperplasia after vascular injury. [29,30,71–73] In our study, accumulation of LC3II protein, reduction in p62 protein degradation, and drastic increase in autophagosome to autolysosome ratio response to TMAO in vivo and in vitro, indicating that TMAO impaired clearance of autophagosomes and blocked autophagic flux. These data verified that autophagic flux was crucial for vascular remodeling after balloon injury, as similarly described by previous studies.

Beclin1, a key regulator of autophagy, can regulate both autophagosome synthesis and autophagosome maturation. [71] Besides, Beclin1 can limit autophagosome clearance and cause excessive autophagosomes accumulation, contributing to cardiomyocyte death. [39,74,75] Similarly, we found that TMAO-induced Beclin1 up-regulation promoted the accumulation of autophagosomes in RAOECs. This finding suggested that over expression of Beclin1 by TMAO induced massive autophagosome formation, and insufficient fusion of excessive autophagosomes with lysosomes, leading to dysfunction of autophagosomal degradation. Nevertheless, TMAO was found to have little effect on lysosomes (Fig. S2A-C in the Data Supplement). However, whether Beclin1 had an effect on fusion between autophagsosomes and autolysosomes remains ambiguous. According to Xiucui et al., Beclin1 can suppress the fusion of autophagy-lysosome and impaired autophagosome processing. [39] The impaired autophagic flux attenuated reendothelialization and ultimately led to neointimal hyperplasia. In the present study, the knockdown of Beclin1 restored TMAO-impaired autophagic flux and reversed the adverse effects of TMAO on RAOECs and balloon-injured vessels. Additionally, in some other studies, Beclin1 was also found to regulate ECs inflammation, oxidative stress, and permeability. [40,41,76].

In conclusion, this study demonstrated that TMAO-induced impairment of autophagic flux aggravated neointimal hyperplasia via activating Beclin1 signaling pathway after balloon injury. Hence, regulation of TMAO metabolism may become a promising strategy against vascular remodeling in various cardiovascular diseases in the future.

Financial support

This study was supported by the National Natural Science Foundation of China (NO.82070247, NO.82000249) and Guangdong Basic and Applied Basic Research Foundation (NO.2019A 1515010899 , NO.2020A1515010288 , NO. 2021A1515220101 , NO.2022A151501 2263)

CRediT authorship contribution statement

Qingqing Hong: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Dongdong Que:** Formal analysis, Investigation. **Chongbin Zhong:** Methodology, Writing – review & editing. **Guanlin Huang:** Methodology. **Weicheng Zhai:** Methodology. **Deshu Chen:** Resources. **Jing Yan:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Pingzhen Yang:** Supervision, Funding acquisition, Writing – review & editing.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank all the individuals who participated in the present study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the

online version at doi:10.1016/j.biopha.2022.113639.

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