Tongxinluo inhibits vascular inflammation and neointimal hyperplasia through blockade of the positive feedback loop between miR-155 and TNF-α

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VASCULAR REMODELING DISEASES, such as restenosis, hypertension, and atherosclerosis, are characterized by the growth and migration of smooth muscle cells (SMCs), proliferation of endothelial cells, and the inflammatory response of macrophages. This histological remodeling results in increased media wall thickness and neointimal formation. Several microRNAs (miRs) have been identified to be involved in vascular inflammation and remodeling. miR-155 and miR-143/145 have been shown to be protective during vascular remodeling, whereas miR-21, and miR-126 have been shown to be involved in vascular inflammation and atherosclerosis. MicroRNA-155 (miR-155) is involved in vascular inflammation and atherosclerosis. However, a direct relationship between TXL and miR-155 in the development of vascular inflammation and remodeling had not yet been shown. The objective of the present study was to investigate whether TXL exerts an inhibitory effect on the vascular inflammatory response and neointimal hyperplasia by regulating miR-155 expression. Using the carotid artery ligation model in mice, we have shown that TXL dose dependently inhibited neointimal formation and reduced the vascular inflammatory response by inhibiting inflammatory cytokine production and macrophage infiltration. miR-155 was induced by carotid artery ligation, and neointimal hyperplasia was strongly reduced in miR-155−/− mice. In contrast, miR-155 overexpression partly reversed the inhibitory effect of TXL on neointimal hyperplasia. In bone marrow-derived macrophages, miR-155 and TNF-α formed a positive feedback loop to promote the inflammatory response, which could be blocked by TXL. Furthermore, TXL increased Akt1 protein expression and phosphorylation in TNF-α-stimulated marrow-derived macrophages, and knockdown of Akt1 abrogated the TXL-induced suppression of miR-155. In conclusion, TXL inhibits the vascular inflammatory response and neointimal hyperplasia induced by carotid artery ligation in mice. Suppression of miR-155 expression mediated by Akt1 and blockade of the feedback loop between miR-155 and TNF-α are important pathways whereby TXL exerts its vasoprotective effects.

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g kg⁻¹day⁻¹), and high dose (1.5 g kg⁻¹day⁻¹) beginning 3 days before ligation injury and continuing for 7, 14, or 21 days thereafter. Ligated animal without TXL treatment received vehicle (water) at an equivalent amount. At specified time points after surgery, all animals were anesthetized and perfused with cold PBS, and tissues were harvested for RNA, morphology, or histological analysis.

**Morphology analysis.** At 7, 14, or 21 days after carotid artery ligation, mice were euthanized and perfused with PBS for 5 min followed by 4% paraformaldehyde for 3 min through the left ventricle under physiological pressure. Carotid arteries were excised and embedded in paraffin. Four-micrometer cross-sections were prepared for hematoxylin and eosin staining, covering the area 3 mm proximal to the ligation site. The neointimal area and intima-to-media ratio were calculated using Image-Pro Plus Analyzer (version 5.1) software (Media Cybernetics, Silver Spring, MD) in a blinded manner. For each section, six random noncontiguous microscopic fields were examined.

**RNA isolation and quantitative RT-PCR.** Total RNA was isolated from carotid arteries and cultured BM-derived macrophages (BMMs) with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Quantitative RT-PCR for the quantification of miR-155 and primary transcript (pri-)miR-155 was performed according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA) in two steps using primer sets (Table I). The neointimal area and intima-to-media ratio were calculated using Image-Pro Plus Analyzer (version 5.1) software (Media Cybernetics, Silver Spring, MD) in a blinded manner. For each section, six random noncontiguous microscopic fields were examined.

**RESULTS**

**TXL inhibits neointimal hyperplasia induced by carotid artery ligation.** Morphometric analysis showed that neointimal hyperplasia was observed at 7 days after vessel ligation and was well developed by 14 days. By 21 days, the neointimal area accounted for >70% of the carotid arterial wall thickness (Fig. 1A). Compared with the ligated group, carotid arterial wall thickness was significantly decreased in the three TXL-treated groups, and both the intima-to-media ratio and intima area were lower than in the ligated group at all three time points (Fig. 1, B and C). Moreover, the inhibitory effect of TXL on neointimal formation was more significant in moderate- and high-dose TXL groups than that in the low-dose TXL group. These results suggest that TXL dose dependently inhibits neointimal formation induced by carotid artery ligation.
TXL inhibits macrophage infiltration, vascular SMC proliferation, and inflammatory cytokine production induced by carotid artery ligation. To assess whether the inhibitory effect of TXL on neointimal formation is related to its anti-inflammation, we determined some cytokines closely related with proinflammatory response and neointimal formation. Quantitative RT-PCR showed a significant increase in PDGF-BB, TNF-α, and IL-1β mRNA in ligated vessels at 21 days after ligation, reaching 6.27-, 16.78-, and 2.07-fold above control levels, respectively. Moderate-dose TXL markedly suppressed carotid artery ligation-induced upregulation of PDGF-BB, TNF-α, and IL-1β mRNA in ligated arteries (Fig. 2A). Next, we identified macrophage infiltration and vascular SMC (VSMC) proliferation by immunofluorescence staining. The results showed that macrophages infiltrated into the neointima of ligated arteries were readily detectable at 21 days after carotid artery ligation with proliferation of a large number of VSMCs, whereas they were barely observed in the neointima in the moderate-dose TXL-treated group, similar to unligated carotid arteries (Fig. 2B). Consistent with mRNA expression results, protein expression of TNF-α and IL-1β as assessed by immunohistochemical staining was significantly elevated in the neointima at 21 days after ligation compared with unligated arteries, but moderate-dose TXL treatment inhibited carotid artery ligation-induced upregulation of TNF-α and IL-1β protein (Fig. 2C). These data indicate that a large number of
macrophages have infiltrated into the neointima of ligated vessels at 21 days after ligation, with an aggravation of VSMC proliferation, and that TXL treatment inhibits macrophage infiltration and VSMC proliferation and decreases the local inflammatory response induced by carotid artery ligation.

**TXL suppresses miR-155 expression induced by carotid artery ligation.** To examine whether the inhibitory effect of TXL on neointimal hyperplasia and macrophage infiltration is related to its regulation of miR-155, we determined the effect of carotid artery ligation on miR-155 expression and whether TXL affected the expression of miR-155 at 7, 14, and 21 days after ligation. Quantitative RT-PCR showed that miR-155 was upregulated in ligated arteries at 14 and 21 days compared with unligated vessels. Importantly, moderate-dose TXL treatment significantly suppressed the expression of miR-155 induced by carotid artery ligation (Fig. 3, A–C). These results indicate that miR-155 is implicated in neointimal formation induced by carotid artery ligation.
carotid artery ligation and that TXL has an inhibitory effect on miR-155 expression.

**miR-155 deletion protects against neointimal hyperplasia induced by carotid artery ligation, and TXL exerts its inhibitory action on neointimal formation by suppressing miR-155 expression.** To examine whether miR-155 is required for neointimal formation induced by carotid artery ligation, we ligated carotid arteries of miR-155−/− and WT mice. As shown in Fig. 4, A–C, marked neointimal hyperplasia was observed in ligated carotid arteries of WT mice on 21 days after ligation, which was strongly reduced in miR-155−/− mice, suggesting that deletion of miR-155 protects against neointimal hyperplasia induced by carotid artery ligation. As expected, neointimal formation was further reduced in ligated carotid arteries of miR-155−/− mice treated with TXL (Fig. 4, A–C). To further define whether the inhibitory effect of TXL on neointimal formation is related to its suppression of miR-155 expression, we then introduced Ad-miR-155 into WT mice through tail vein injection to overexpress miR-155. Quantitative RT-PCR showed that miR-155 expression was increased by 3.5-fold after Ad-miR-155 injection in ligated arteries of TXL-treated mice and was barely detected in ligated arteries of miR-155−/− mice (Fig. 4D). Neointimal thickness was significantly increased after miR-155 overexpression regardless of TXL treatment (Fig. 4, A–C), indicating that overexpression of miR-155 partly reversed the inhibitory effect of TXL on neointimal hyperplasia. Accordingly, macrophage infiltration and VSMC proliferation were barely observed in the neointima after carotid artery ligation in miR-155−/− mice compared with WT mice. Meanwhile, in TXL-treated WT mice, Ad-miR-155-infected arteries showed increased macrophage infiltration and VSMC proliferation compared with Ad-null-infected arteries after carotid artery ligation (Fig. 4E).

Next, we examined mRNA expression levels of TNF-α and IL-1β in the different groups of mice. Quantitative RT-PCR revealed that the mRNA level of TNF-α in ligated arteries of miR-155−/− mice was reduced by 11% of WT mice. In TXL-treated miR-155−/− mice, TNF-α mRNA levels were reduced to 20% of TXL-treated WT mice. Importantly, TNF-α mRNA levels were increased 2.2-fold in ligated arteries of Ad-miR-155-infected mice compared with Ad-null-infected mice regardless of TXL treatment, suggesting that miR-155 might mediate TNF-α mRNA expression and that miR-155 overexpression declines the inhibitory effect of TXL on TNF-α expression (Fig. 4F). In contrast, although a 50% reduction of IL-1β mRNA was observed in ligated arteries of miR-155−/− mice, no significant difference in IL-1β mRNA levels was detected between miR-155−/− and WT mice when these mice were treated with TXL. Moreover, after carotid artery ligation, miR-155 overexpression did not affect IL-1β mRNA expression in ligated arteries regardless of TXL treatment (Fig. 4G). These results suggest that the inhibitory effect of TXL on IL-1β expression is not through suppression of miR-155 expression.

**miR-155 and TNF-α form a positive feedback loop to promote the macrophage inflammatory response, and TXL blocks this feedback pathway.** The macrophage inflammatory response is a key event for the initiation and progression of vascular remodeling (11, 26). Because we found that macrophage infiltration was strongly associated with vascular inflammation induced by carotid artery ligation and that TXL could improve artery structure after ligation as well as that deletion or overexpression of miR-155 markedly affected TNF-α expression in ligated arteries, we sought to determine whether there was a regulatory relationship between miR-155 and TNF-α in the inflammatory response of macrophages. First, mouse BMMs were stimulated with TNF-α or IL-1β. Quantitative RT-PCR showed that miR-155 expression was increased by 2.5-fold after TNF-α treatment, whereas TXL treatment suppressed the expression of miR-155 induced by TNF-α in a dose-dependent manner (Fig. 5A). On the other hand, no significant upregulation of miR-155 expression was observed after IL-1β treatment, and TXL treatment did not significantly affect miR-155 expression (Fig. 5B). These results suggest that TNF-α, but not IL-1β, can markedly induce miR-155 expression in BMMs. We next tested whether miR-155 also affected the expression of TNF-α or IL-1β. Quantitative RT-PCR revealed that overexpression of miR-155 mediated by an adenoviral vector in BMMs increased the expression of TNF-α by sixfold over that of Ad-null-infected BMMs, whereas BMMs from miR-155−/− mice had a much lower level of TNF-α expression compared with that of BMMs from WT mice (Fig. 5C). Similarly, overexpression of miR-155 in BMMs significantly increased the release of TNF-α into the
medium, but BMMs from miR-155−/− mice released less TNF-α into the culture medium compared with WT mice (Fig. 5D). However, overexpression or deletion of miR-155 did not affect the expression of IL-1β (Fig. 5, E and F). TXL pretreatment reduced the upregulation of TNF-α expression induced by miR-155 overexpression, with the TNF-α mRNA level being reduced to 31% of the control and the TNF-α protein level being decreased to 78% of the control. Moreover, BMMs from miR-155−/− mice also showed a low level of TNF-α expression regardless of TXL treatment (Fig. 5, G and H). Taken together, these results suggest that miR-155 and TNF-α form a positive feedback loop to promote the macrophage inflammatory response in mice and that TXL has an inhibitory effect on this feedback pathway. Furthermore, pri-miR-155

Fig. 4. miR-155 deletion protects against neointimal hyperplasia induced by carotid artery ligation, and TXL exerts its inhibitory effect on neointimal formation via suppression of miR-155 expression. A: neointimal hyperplasia 21 days after carotid artery ligation. Representative sections of hematoxylin and eosin-stained arterial sections from different groups of ligated vessels, including wild-type (WT) mice treated with or without moderate-dose TXL, miR-155−/− mice treated with or without moderate-dose TXL, and WT mice injected with Ad-null or Ad-miR-155 and treated with moderate-dose TXL, and unligated vessels from WT and miR-155−/− mice, are shown. Magnification: ×200. B: morphometric quantification of the IM ratio. C: morphometric quantification of the intima area. D: quantitative RT-PCR of miR-155 in ligated arteries of Ad-null- or Ad-miR-155-injected mice treated with TXL as well as WT and miR-155−/− mice (n = 6 in each group). E: immunofluorescence for Mac-2 (red), SMA-α (green), and DAPI (blue) in ligated arteries from WT and miR-155−/− mice as well as TXL-treated mice injected with Ad-null or Ad-miR-155. Bars = 50 μm. F: quantitative RT-PCR of TNF-α in different groups of mice. G: quantitative RT-PCR of IL-1β in different groups of mice. Data represent means ± SE; n = 9 in each group. *P < 0.05 and **P < 0.01 vs. WT mice; #P < 0.05 and ##P < 0.01 vs. miR-155−/− mice; $P < 0.05 and $$P < 0.01 vs. Ad-null-injected mice treated with moderate-dose TXL.
expression was also upregulated after TNF-α treatment, whereas TXL treatment significantly suppressed the expression of pri-miR-155 induced by TNF-α, suggesting that TXL downregulates miR-155 at the transcriptional level (Fig. 5I).

Akt1 is required for TXL-induced suppression of miR-155 expression. Because Akt1 negatively regulates miR-155 expression in macrophages (2), we sought to determine whether TXL suppressed miR-155 expression through Akt1 signaling in BMMs. As shown in Fig. 6A, there was a slight decrease in levels of p-Akt upon TNF-α stimulation, but TXL pretreatment markedly increased Akt phosphorylation. Interestingly, total Akt1 also markedly increased by TXL pretreatment for 2 h, suggesting that the increased p-Akt levels may be due to the upregulation of total Akt1 protein levels by TXL pretreatment. Next, we knocked down Akt1 by transfection of BMMs with Akt1-specific siRNA and examined the effect of TXL on miR-155 expression. Akt1 knockdown abrogated the inhibitory effect of TXL on miR-155 expression induced by TNF-α (Fig. 6B), indicating that TXL inhibits miR-155 expression by upregulating Akt1 in BMMs.

TXL posttreatment still inhibits neointimal hyperplasia induced by carotid artery ligation. TXL was administrated intragastrically at a moderate dose beginning 3 days after ligation injury and continued for 21 days thereafter. Morphometric analysis showed that TXL posttreatment still inhibited neointimal hyperplasia induced by carotid artery ligation, although
the suppressive effect of TXL became less robust (Fig. 7, A–C).

DISCUSSION

The major findings of the present study were that 1) TXL dose dependently inhibited neointimal formation induced by carotid artery ligation, 2) TXL reduced the local inflammatory response in ligated vessels by inhibiting inflammatory cytokine production and macrophage infiltration, 3) miR-155 deletion protected against neointimal hyperplasia induced by carotid artery ligation and TXL exerted its inhibitory action on neointimal formation by suppressing miR-155 expression, 4) miR-155 and TNF-α formed a positive feedback loop to promote the macrophage inflammatory response and TXL blocked this feedback pathway, and 5) Akt1 mediated TXL-induced suppression of miR-155 expression.

Inflammation plays a key role in the development of vascular remodeling such as atherosclerosis, restenosis after angioplasty, and bypass graft failure (5, 6, 26, 33). Intimal thickening caused by an accumulation of VSMCs and macrophages is a central feature of the vascular remodeling process (9).

A previous study (34) has demonstrated that TXL inhibited oxidized LDL-induced maturation of human dendritic cells and secretions of IL-12 and TNF-α through activation of the peroxisome proliferator-activated receptor-γ pathway. Treatment with TXL significantly reduced serum levels of monocyte chemoattractant protein-1, high-sensitivity CRP, IL-8, IL-18, MMP-1, and P-selectin and protected against plaque inflammation in an atherosclerosis rabbit model (8). Furthermore, TXL dose dependently lowered protein expression levels of lectin-like oxidized LDL-R1, MMP-1, MMP-3, and NF-κB in plaques of a rabbit model of vulnerable plaques (39). The above findings indicate that TXL is a potent drug for anti-inflammation. However, it remains unclear whether and how TXL modulates neointimal hyperplasia via inhibition of vascular inflammation.

Blood flow cessation by complete ligation of the vessel near the carotid bifurcation can produce flow-induced vascular remodeling with neointimal formation in the mouse carotid artery (19). The reduction in shear stress increases endothelial cell apoptosis and proliferation and induces a proinflammatory phenotype characterized by adhesiveness of macrophages (1). Accordingly, low shear stress due to disturbed flow is typically found in regions of the arterial tree, such as branching points or the outer curvature of the aortic arch, which are highly susceptible for atherosclerosis (10). In the present study, we used this model to observe the role of TXL in protecting against flow-induced vascular remodeling in the mouse and found that TXL inhibited the neointimal hyperplasia induced by carotid artery ligation regardless of pretreatment or posttreatment.

Growth factors and cytokines produced by the injured endothelium and inflammatory cells trigger the migration and proliferation of SMCs into the intima. A previous study (32) has shown that low shear stress induced by carotid artery ligation promoted TNF-α and IL-1 expression and neointimal hyperplasia and that mice lacking either functional TNF-α or IL-1 develop less neointima than WT control mice. Furthermore, increased levels of TNF-α preceded the migration of VSMCs into the intima in a balloon-injured rat aorta model by several days (18). Similarly, we found that mRNA expression levels of TNF-α and IL-1β in ligated carotid arteries were significantly increased compared with those in unligated vessels. Particularly, TNF-α mRNA in ligated vessels at 21 days after ligation was increased 16.78-fold above the control level. Meanwhile, PDGF-BB was also elevated after ligation, which is one of the key modulators of the SMC phenotype and proliferation (38), indicating that an increased inflammatory response in the ligated artery might also lead to a increase in growth factor secretion, which further promotes cell prolifer-
Our results showed that moderate-dose TXL markedly suppressed carotid artery ligation-induced upregulation of TNF-α, IL-1β, and PDGF-BB expression in the ligated artery. Macrophage infiltration plays a pivotal role in the pathogenesis of vascular remodeling. Systemic inactivation and depletion of macrophages by liposomal clodronate reduced neointimal hyperplasia and restenosis in hypercholesterolemic rabbits and rats (11). Our immunohistochemistry results also confirmed that macrophages infiltrated the neointima of ligated arteries at 21 days after carotid artery ligation with proliferation of a large number of VSMCs. Meanwhile, TNF-α and IL-1β levels were also significantly elevated in the neointima at 21 days after ligation. Whereas TXL treatment inhibited macrophage infiltration, VSMC proliferation and inflammatory cytokine production were induced by carotid artery ligation.

miR-155 promotes the inflammatory response of macrophages by modifying their inflammatory capacity (2, 3, 28) and is upregulated in atherosclerotic lesions of humans and mice but reduced in the circulation of patients with coronary artery disease (15, 28). miR-155 can promote and inhibit inflammatory macrophage activation in vitro by targeting several mediators of inflammatory signaling, such as SHIP1, SOCS1, SMAD2, and TAB2 (2, 7, 24, 25). In vivo, suppression of Bcl6 by miR-155 increases the inflammatory response in lesional macrophages and thereby promotes lesion formation (28). On the other hand, unidirectional high shear stress upregulates miR-155 in endothelial cells in which miR-155 limits the inflammatory response to ANG II by suppressing ANG II type 1 receptors and protein C-ets-1 (41). Furthermore, miR-155−/−/ApoE−/− (double knockout) mice developed fewer atherosclerotic lesions in the aortic root with reduced neutral lipid content and macrophages (13). Although miR-155 is expressed in lesional SMCs, the development of atherosclerosis was not affected in miR-155−/−/ApoE−/− mice harboring miR-155+/+ BMMs, suggesting that miR-155 expressed in vascular cells does not play a crucial role in atherogenesis (28). In contrast, hematopoietic deficiency of miR-155 enhanced atherosclerotic plaque development and decreased plaque stability by increased myeloid inflammatory cell recruitment to the plaque in LDL-R−/− mice (12). The opposite effects of miR-155 on lesion formation may depend on the stage of atherosclerosis.

In the present study, we found that miR-155 was upregulated in ligated arteries and that neointimal hyperplasia was strongly reduced in miR-155−/− mice compared with WT mice, suggesting that deletion of miR-155 protects against neointimal hyperplasia induced by carotid artery ligation. These findings indicate an important role of miR-155 in mediating neointimal formation. We further defined whether the inhibitory effect of TXL on neointimal formation is related to its regulation of miR-155 expression. We found that TXL treatment significantly suppressed the expression of miR-155 induced by carotid artery ligation. Importantly, overexpression of miR-155 partly reversed the inhibitory effect of TXL on neointimal hyperplasia and macrophage infiltration. By collecting these results, we show, for the first time, a crucial role of miR-155 in...
neointimal formation induced by carotid artery ligation and demonstrated that TXL exerted its inhibitory action on neointimal formation partly by suppressing miR-155 expression.

Interestingly, the mRNA level of TNF-α in ligated arteries of miR-155−/− mice was markedly reduced compared with that of WT mice and was increased 2.2-fold in ligated arteries infected with Ad-miR-155 regardless of TXL treatment, suggesting that miR-155 can positively regulate TNF-α expression and that miR-155 overexpression abrogates the inhibitory effect of TXL on TNF-α expression. In contrast, although the mRNA level of IL-1β in ligated arteries of miR-155−/− mice was reduced by 50% of WT mice, there was no significant difference between miR-155−/− and WT mice when these mice were treated with TXL. Moreover, after carotid artery ligation, miR-155 overexpression did not affect IL-1β mRNA expression in ligated arteries regardless of TXL treatment. These results suggest that the inhibitory effect of TXL on IL-1β expression is not through suppression of miR-155 expression.

Our study in vivo implicated a potential regulatory relationship between miR-155 and TNF-α in the inflammatory response. Previous studies (30, 35) have shown that TNF-α could upregulate miR-155 expression in macrophages and endothelial cells. However, TNF-α levels were decreased in alcohol-treated macrophages after inhibition of miR-155, and miR-155 overexpression increased TNF-α production by increasing the TNF-α mRNA half-life in human macrophages (4, 31). In the present study, TNF-α markedly induced miR-155 expression in BMMs. On the other hand, miR-155 deletion decreased, whereas miR-155 overexpression increased, TNF-α expression in BMMs. These results suggest that miR-155 and TNF-α might form a positive feedback loop to promote the macrophage inflammatory response in mice. We found that TXL treatment suppressed the expression of miR-155 induced by TNF-α in a dose-dependent manner and reduced the upregulation of TNF-α expression induced by miR-155 overexpression. These findings indicated that TXL has a inhibitory effect on this feedback pathway revealing a mechanistic role of TXL in anti-inflammation. However, IL-1β did not induce upregulation of miR-155 expression, and overexpression or deletion of miR-155 also did not affect IL-1β expression in BMMs, consistent with our results in vivo, suggesting that miR-155 has no effect on IL-1β expression.

Akt1 differentially regulates miRNAs, including Let-7e, miR-155, miR-181c, and miR125b, in LPS-stimulated macrophages (2). Akt1 ablation in mice shows a proinflammatory phenotype, resulting in enhanced atherosclerosis (14), Akt1 suppression by miR-342-5p induced proinflammatory mediators, such as inducible nitric oxide synthase and IL-6, in macrophages via the upregulation of miR-155 during atherosclerosis. Moreover, upregulation of miR-155 induced by LPS/interferon-γ could be abolished by an miR-342–5p inhibitor but then subsequently restored by siRNA-mediated Akt1 suppression in macrophages (36). As expected, TXL pretreatment increased Akt1 protein expression and phosphorylation in TNF-α-stimulated BMMs, and the siRNA-mediated suppression of Akt1 abrogated the inhibitory effect of TXL on miR-155 expression induced by TNF-α, suggesting that Akt1 is required for TXL-induced suppression of miR-155 expression.

However, TXL-mediated cardiovascular protection is not only via anti-inflammatory effect on macrophages but also via an improvement of endothelial function. For example, TXL protected palmitic acid-induced endothelial damage by initiating AMP-activated protein kinase-mediated activation of the thioredoxin antioxidant system (40). TXL activated the JNK/c-Jun/heme oxygenase-1 pathway to improve endothelial function in overfatigued rats (22). Furthermore, TXL upregulated eNOS expression via the phosphatidylinositol 3-kinase/Akt/hypoxia-inducible factor-dependent signaling pathway to promote endothelium-dependent vasodilation (21). On the hand, genetic eNOS deficiency increases the expression of proinflammatory cytokines and macrophage infiltration in adipose tissue, ischemic muscles, and renal injuries (16, 17, 27). On the other hand, eNOS is a direct target of miR-155 in endothelial cells (35). Therefore, TXL-mediated protection against vascular inflammation and remodeling may be also through the eNOS pathway and subsequent reductions in macrophage infiltration.

A major limitation of the present study is the lack of characterization of the targets of miR-155 in the context of TXL treatment, which is already as a part of continuing efforts in our next project. Another limitation is that although the components of TXL are clear, the active ingredients of TXL and their interactions remain to be clarified.

In conclusion, TXL inhibits the vascular inflammatory response and neointimal hyperplasia induced by carotid artery ligation in mice. Suppression of miR-155 expression via upregulation of Akt1 and blockade of the feedback loop between miR-155 and TNF-α is one of the mechanisms underlying the vasoprotective effects of TXL (Fig. 8).

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Authors contributions: R.-n.Z., B.Z., and J.-k.W. conception and design of experiments; R.-n.Z. and B.Z. analyzed data; R.-n.Z., B.Z., X.-h.Z., and J.-k.W. interpreted results of experiments; R.-n.Z. prepared figures; R.-n.Z. drafted manuscript; R.-n.Z. and J.-k.W. edited and revised manuscript; R.-n.Z. and J.-k.W. approved final version of manuscript.

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