Vascular restenosis reduction with platelet membrane coated nanoparticle directed M2 macrophage polarization

Highlights
- Targeted immune regulation can reduce restenosis after vascular injury
- IL10-PNP can target and regulate M2 macrophages polarization in vivo
- Vascular restenosis reduction mediated by IL10-PNP requires less cell membrane quantity
Vascular restenosis reduction with platelet membrane coated nanoparticle directed M2 macrophage polarization

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SUMMARY

Vascular restenosis is the main factor affecting the prognosis of angioplasty in cardiovascular diseases, and inflammation is a central link in the progression of restenosis. Previous research that applies interleukin 10 (IL10) nanoparticles can effectively regulate local inflammation, but their targeted delivery efficacy remains to be improved. In this study, IL10 nanoparticles were successfully prepared and then coated by a preactive platelet membrane. The ability to target and regulate macrophage polarization has been demonstrated, thereby regulating smooth muscle cell and endothelial cell functions. In vivo experiments were carried out in a carotid artery injury model and verified the above functions and the effect on inhibiting vascular restenosis. Immune regulation-based platelet membrane coated nanoparticle loaded with IL10 proved to be an excellent candidate for targeting vascular injury and holds promise as an innovative drug delivery system for suppressing vascular restenosis.

INTRODUCTION

Atherosclerotic cardiovascular diseases remain the most serious health problem worldwide, with high rates of mortality and morbidity (Cannon, 2013). One of the most common treatments for patients suffering from these diseases is interventional angioplasty to reconstruct the vascular patency. However, angioplasty causes vascular injury and hence may lead to restenosis, which has a high reintervention rate and affects the prognosis of patients (Bønaa et al., 2016; Jukema et al., 2011). In the process of interventional angioplasty, the patency of the vasculature was reconstructed, while the vessel wall was injured, causing local de-endothelialization damage, which recruits immune cells and platelets. Inflammatory cytokines secreted by immune cells and platelets such as platelet-derived growth factor-BB, interleukin-1, and so forth, will cause proliferation and migration functions of smooth muscle cells (SMCs) and eventually lead to vascular restenosis (Clare et al., 2022). Vascular restenosis is closely related to the inflammatory reaction to injury. Macrophages are a requisite component for inflammatory reactions and have massive infiltration in the restenosis site (McDonald et al., 2015; Zhang et al., 2014a). Macrophage polarization plays an important role in the process of restenosis. Depending on the cytokines secreted and their functions, macrophages are mainly divided into two polarized types, including classically activated macrophages (M1 phenotype), also known as pro-inflammatory macrophages, and alternatively activated macrophages (M2 phenotype), also known as anti-inflammatory macrophages (Orecchioni et al., 2019; Yang et al., 2020). Vascular injury mediates macrophage polarization to the M1 phenotype that secretes cytokines such as IL-1β, TNF-α, and IL-6 induces SMCs transformation to the secretory phenotype, which is active in proliferation and migration and eventually leads to restenosis (Xing et al., 2022). On the other hand, the M2 phenotype reduces vascular restenosis by (1) reducing the secretion of inflammatory cytokines thereby inhibiting the secretory phenotype transformation of smooth muscle cells; (2) promoting endothelial repair to reduce inflammatory cell infiltration and inhibit smooth muscle cell proliferation and migration; (3) exosome-induced regulation in the proliferation and migration of smooth muscle cell (Koelwyn et al., 2018; Yan et al., 2020). Therefore, targeted stimulation of M2 macrophage polarization effectively reduces vascular restenosis.

Platelets have long been recognized for their role in hemostasis. In recent years, the important role of platelets in inflammatory reactions has been revealed (Margraf and Zarbock, 2019). After vascular injury, activated platelets adhere rapidly to the injury site via several adhesion receptors (Chandrasekar and Tanguay, 2000). Meanwhile, in patients with cardiovascular diseases, increased circulating platelet-leukocyte
aggregates have been demonstrated, which is a protagonist of vascular inflammation (Totani and Evangelista, 2010). Platelets directly interact with immune cells through P-selectin, CD42b, and CD40L to regulate their functions (Zann et al., 2018; Li et al., 2019; Wang et al., 2017; Willecke et al., 2014). The structure and function of CD42b on the surface of activated platelets would change (Li et al., 2019; Quach and Li, 2020), accompanied by the membrane surface expression of P-selectin from platelet α-granules (Badlou et al., 2006) and CD40L from the cytoplasm (Hermann et al., 2001). Active platelets bind to macrophages, and platelet-macrophage aggregations play an important role in the process of restenosis after vascular injury (Barrett et al., 2019; Wang et al., 2005). Owing to the targeted binding effect of platelets on macrophages in local vascular injury, it would be an effective way to target macrophages by mimicking the adhesive property of platelets.

With the development of nanotechnology, nanoparticles (NP) drug delivery system has been widely considered owing to their high targeting, effectiveness, good pharmacokinetic and pharmacodynamic characteristics (Kang et al., 2015). Modification of its surface can effectively improve its biological efficiency (Hou et al., 2022). Natural cell membrane-coated nanoparticles are being explored to obtain cell-like properties for nanoparticle functionalization (Fang et al., 2018; Le et al., 2021). They preserve the natural structure of cell membranes with prolonged circulation time and improved selective targeting delivery ability (Fang et al., 2017). With a set of glycoproteins uniquely expressed on the platelet surface, platelet membrane-coated nanoparticles (PNPs) have shown satisfactory results in the diagnosis and treatment of many diseases, such as vascular diseases, infectious diseases, and cancer (Chen et al., 2021; Huang et al., 2021; Wang et al., 2020). Owing to the adhesion function of platelets to macrophages after vascular injury, platelet-membrane-coated nanoparticles have the potential to reduce restenosis after vascular injury by specifically targeting regulating macrophage polarization.

Interleukin 10 (IL10) is a broad-spectrum anti-inflammatory cytokine with immunoregulatory functions (de Vries, 1995). Clinical trials of recombinant human IL10 for various inflammatory diseases, such as inflammatory bowel disease, chronic hepatitis C, psoriasis, and rheumatoid arthritis are underway (Asadullah et al., 2003). IL10 is known to regulate M2 macrophage polarization in multiple ways (Ip et al., 2017). However, the systemic application of IL10 results in side effects such as anemia and thrombocytopenia (Asadullah et al., 2003; Fredman et al., 2015). In previous studies, IL10 nanoparticles were prepared to inhibit the local inflammatory response of atherosclerotic lesions (Kamaly et al., 2016; Kim et al., 2020). However, its regulatory effect on local immune cells has not been elucidated, and its targeted delivery capability has the potential to be further improved.

In this study, we used IL10 as an agent being coated and discovered platelet membrane-coated poly (DL-lactide-co-glycolide) (PLGA) nanoparticles (NPs) as a targeted drug delivery system by mimicking the adhesive property of platelets to macrophages in vascular restenosis. The regulation of PNP encapsulated IL10 (IL10-PNP) on macrophage polarization was evaluated in vitro. Then, the anti-restenosis effect of IL10-PNP was assessed in a rat model of angioplasty-induced vascular injury.

RESULTS

Fabrication and characterization of IL10-NP and IL10-PNP

In the present study, a platelet-like delivery platform to target vascular injury was synthesized by fusing platelet membranes to the surface of PLGA nanoparticles by ultrasound. Transmission electron microscope (TEM) and dynamic light scattering (DLS) were performed to characterize the structure and fabrication efficiency of the nanoparticles. TEM results demonstrated that compared with IL10-NPs, IL10-PNPs were adequately coated with platelet membranes and possessed a core-shell structure (Figures 1A and 1B). Coomassie Blue staining revealed similar membrane protein retention and enrichment between platelet membrane vesicles and PNPs (Figure 1C). Western blotting revealed comparable expression of membrane proteins bound to macrophages, including P-selectin, CD42b, and CD40L, between platelet membrane vesicles and PNPs (Figure 1D). Compared with IL10-NP, IL10-PNP displayed a slight increase in size (from 131.22 ± 0.75 nm to 164.41 ± 1.36 nm) and surface zeta potential (from −29.97 ± 0.15 mV to −25.53 ± 0.81 mV) (Figures 1E and 1F). After storage in deionized water for 7 days, the size of IL10-PNP was comparable without any significant change (from 164.41 ± 1.36 nm to 164.90 ± 0.64 nm) (Figure 1G), indicating that IL10-PNP has good stability. Under experimental conditions, the encapsulation efficiency of IL10 was 80.77 ± 0.67% and 80.30 ± 0.70% for IL10-NP and IL10-PNP, respectively (Figure 1H). The loading capacity of IL10 was 2.52 ± 0.11% and 2.53 ± 0.11% for IL10-NP and IL10-PNP, respectively (Figure 1I). The release profiles of IL10 from IL10-NP and IL10-PNP were evaluated by incubating at 37°C in PBS, then
collecting and measuring the released protein at different intervals for up to 240 h with IL-10 ELISA. IL10-NP and IL10-PNP both showed controlled-release profiles for IL10. IL10-NP released IL10 faster in the first 40 h, both IL10-NP and IL10-PNP peaked at 100 h, reaching 88.26 ± 0.52% and 86.56 ± 0.41% of the total encapsulated IL10, respectively (Figure 1J).

**Binding function of IL10-PNP to macrophages in vitro**

The binding function of IL10-PNP to macrophages was assessed by detecting the expression of the platelet membrane glycoprotein CD61 on macrophages. CD61 positivity on the surface of macrophages indicated the binding of IL10-NP or platelets to macrophages. After 24 h of co-culture, immunofluorescence staining showed no significant difference in fluorescence intensity at different time points (Figures 2A and 2C). And the ratio of CD61⁺ macrophages in the preactive platelet group and the IL10-PNP group showed no significant difference (94.30 ± 0.95% and 95.13 ± 1.52%, respectively) (Figures 2B and 2D), suggesting that the binding ability of IL10-PNP to macrophages was similar to activated platelets which could effectively bind to macrophages.

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**Figure 1. PNP characterization**

(A) Transmission electron micrographs of IL10-NP, and (B) IL10-PNP.

(C) Coomassie staining of platelet vesicles and empty PNP on SDS-PAGE at equivalent protein concentrations.

(D) Western blot analysis of platelet vesicles and PNP for characteristic platelet membrane glycoprotein bound to macrophages.

(E) Size of empty PLGA core, IL10-NP, empty PNP, and IL10-PNP.

(F) Zeta potential of empty PLGA core, IL10-NP, empty PNP, and IL10-PNP.

(G) Diameter of PNP in deionized water on day 0 and day 7.

(H) Encapsulation efficiency and (I) loading capacity of IL10 in IL10-NP, IL10-PNP.

(J) In vitro cumulative release curve of IL10-NP and IL10-PNP incubated at 37°C in PBS. *p < 0.05, **p < 0.01, ***p < 0.001. n = 3.
Regulation efficacy of IL10-PNP on macrophage polarization in vitro

Previous studies and preliminary experiments have demonstrated that platelets can regulate M1 macrophage polarization and have similar effects as lipopolysaccharide (LPS) (Figure S4) (Carestia et al., 2019). To evaluate the regulatory efficacy of IL10-PNP on macrophage polarization in the vascular injury site, platelets were added to the medium to stimulate the inflammatory environment (Figure 3A). The M1
macrophage polarization marker CD86 and M2 macrophage polarization marker CD163 were detected by flow cytometry. In the PBS group, the ratio of M1 macrophages significantly increased (8.78 ± 0.39%) (Figures 3B and 3C), which means platelets contribute to the inflammatory action of macrophages. The ratio of M2 macrophages significantly increased in the IL10 group, IL10-NP group, and IL10-PNP group, and there was no significant difference between the three groups (39.43 ± 1.11% and 36.50 ± 1.06% and 35.97 ± 0.31%, respectively) after 24 h of coculture (Figures 3B and 3D). The application of the same amount of empty PNP did not promote M1 macrophage polarization (Figure S4). IL10-PNP effectively inhibits macrophage inflammation and promotes M2 macrophage polarization. Meanwhile, the cytokines secreted by M1 macrophages (TNF-α, IL-1β, IL-6) significantly increased in the PBS group (Figures 3E-3G). The preparation of nanoparticles and platelet membrane coating had no significant effect on the release and regulation efficacy of IL10 in vitro.

**Figure 3.** Regulation role of IL10-PNP on macrophage polarization in an inflammatory environment simulated in vitro

(A) Schematic diagram of the coculture system.
(B) Flow cytometry detected the polarization of macrophages cultured alone and cocultured with platelets + PBS, platelets + IL10, platelets + IL10-NP, and platelets + IL10-PNP for 24 h.
(C) M1 macrophages and (D) M2 macrophages ratio in different groups. Levels of TNF-α (E), IL-1β (F), and IL-6 (G) in the cell supernatant were measured with ELISA. Data are presented as mean ± SD, ns not significant, *p < 0.05, **p < 0.01, ***p < 0.001, n = 3.
Figure 4. Regulation role of IL10-PNP on SMCs in inflammatory environment simulated by platelets + macrophages coculture system in vitro

(A) Schematic diagram of the coculture system.

(B) Relative expression of osteopontin (OPN) and (C) SM22 in SMCs by RT-qPCR. n = 3.

(D and E) Relative expression of a contractile phenotype related protein in SMCs after 24 h coculture. n = 3.

(F and G) The impact of IL10-PNP on the proliferative ability of SMCs detected by Edu assay. n = 3.
Application of IL10-PNP to the phenotypic transformation and functions of smooth muscle cells

A co-culture system with platelets was established to simulate the inflammatory environment in vitro (Figure 4A). Clinically, the hyperplastic intima is formed by the proliferation and migration of SMCs after phenotypic transformation into the secretory type. SMCs in the PBS group had significantly increased expression level of a secretory phenotype-related gene OPN, and decreased expression level of contractile phenotype-related gene SM22 (Figures 4B and 4C), while IL10, IL10-NP, or IL10-PNP application significantly decreased the expression of OPN (Figure 4B). SMCs' contractile phenotype-related proteins were significantly decreased in the PBS group, while the phenotypic transformation of SMCs was inhibited after IL10, IL10-NP, or IL10-PNP application, (Figures 4D and 4E). The proliferation of SMCs was assessed by CCK-8 assay and Edu assay. Compared with the blank group, SMCs in the PBS group had significantly increased proliferation ability, while the application of IL10, IL10-NP, and IL10-PNP significantly inhibited the proliferation ability of SMCs (Figures 4F-4H). The migration of SMCs was assessed by Transwell assay. Compared with the blank group, SMCs in the PBS group had significantly increased migration ability, and the application of IL10, IL10-NP, and IL10-PNP significantly inhibited the migration of SMCs (Figures 4H and 4I). By simply joining platelets and macrophages to stimulate the local inflammatory reaction, the secretion of inflammatory factors promoted SMCs proliferation and migration, which would further lead to restenosis. With the application of IL10, IL10-NP, or IL10-PNP, macrophage function could be regulated, and the local inflammatory reaction was reduced, hence inhibiting SMCs proliferation and migration. Meanwhile, the preparation of nanoparticles and platelet membrane coating had no significant effect on the regulation of smooth muscle cell proliferation function and phenotypic transformation in vitro.

IL10-PNP on the repair function of endothelial cells

Early repair of endothelial cells after vascular injury could reduce immune cell infiltration and prevent smooth muscle cell hyperproliferation. The repair function of endothelial cells was assessed by tube formation assay and wound healing assay. The tube formation assay showed significantly decreased endothelial cell function in the PBS group and significantly increased function in the IL10 group, IL10-PNP group, and IL10-NP group (Figures 5A and 5B). During the endothelial cell wound healing assay, endothelial cells in the IL10 group, IL10-PNP group, and IL10-NP group healed significantly faster than in the PBS group (relative gap area) (Figures 5C and 5D). IL10 released by PNPs, or NPs suppressed the inflammatory effect of macrophages and enhanced the repair effect of endothelial cells, and platelet membrane coating had no significant effect on the regulation of endothelial cell repair function.

Targeting effect and release function of platelet membrane-coated nanoparticles on vascular injury

To evaluate whether circulating PNP could gather to sites of vascular injury in vivo, DiR-labeled NPs and PNPs were intravenously injected after the vascular injury procedure. 24 h after injection, the fluorescence of the bilateral carotid arteries and major organs, including the heart, liver, spleen, lungs, and kidneys, was examined ex vivo because the rat tissue structure was too thick to obtain a fluorescence signal in vivo. The fluorescence intensity at the site of vascular injury was significantly increased in the PNP group (Figures 6A and 6B), indicating that PNP can gather at the site of vascular injury. The fluorescence intensity of the kidney was lower in the PNP group, indicating that DiR metabolized faster in saline and NPs (Figure 6B). To evaluate the release function of IL10-PNP in vivo, IL10 concentration was measured in different organs 24 h after treatment. IL10 concentration significantly increased in the left carotid artery in the IL10-PNP group compared with the IL10-NP group (Figure 6C). The concentration in other organs slightly increased, without significant difference with the IL10-NP group.

In vivo targeted regulation efficacy of macrophage polarization

The impact of IL10-PNP on macrophage phenotype polarization was next assessed by examining the expression of established M1 and M2 macrophage-associated markers. On Day 14, the injured left common carotid artery was obtained for histological analysis. To evaluate the proportion of macrophages...
Figure 5. Regulation role of IL10-PNP on endothelial cells functions in an inflammatory environment simulated by platelets + macrophages coculture system in vitro
(A) The impact of IL10-PNP on HUVECs function by tube formation assay.
(B) Quantitative analysis of tube formation assay in different groups.
(C) The impact of IL10-PNP on HUVECs repair function by wound healing assay.
(D) The relative gap area of wound healing assay in different groups, values were normalized based on the value of the blank group. n = 3. Data are presented as mean ± SD, ns not significant, *p <0.05, **p <0.01, ***p <0.001. Scale bar = 50 μm.
M1 macrophages (F4/80+/CD86+) and M2 macrophages (F4/80+/CD163+) at the site of vascular injury, tissue sections were co-stained with F4/80 and CD86 or co-stained with F4/80 and CD163. The proportion of macrophages in the saline group, IL10 group, IL10-NP group, and IL10-PNP group was significantly higher than in the sham group (Figures 7 A-7C), indicating that vascular injury could lead to local macrophage infiltration. After IL10-PNP application, the proportion of macrophages was significantly decreased (Figures 7 A-7C). Furthermore, the proportion of M1 macrophages in the saline group and IL10 group were significantly higher than that in the IL10-NP group and IL10-PNP group (Figures 7 A, 7B, and 7D), indicating that in the natural process after vascular injury, macrophages will polarize into the M1 phenotype, which would promote local inflammation, and that the systemic application of 1 μg IL10 is too low a dose to be effective at the site of vascular injury. The proportion of M2 macrophages in the IL10-PNP group was significantly higher than that in the other two groups (Figures 7 A, 7B, and 7E), showing that IL10-PNP could promote the polarization of local macrophages to the M2 phenotype, which would suppress inflammation and promote tissue repair.

**Anti-restenosis role of IL10-PNP**

Fourteen days after balloon injury, the injured left common carotid arteries of rats were harvested, and H&E staining was used to analyze restenosis after vascular injury. The neointima areas in the IL10-PNP group were significantly reduced compared to those in the IL10-NP group, IL10 group, and saline group at 14 days (Figures 8 A and 8D). The ratio of intimal to media (I/M) was measured as an index, and the I/M in the IL10-PNP group was significantly reduced compared to that in the IL10-NP group, IL10 group, and saline group at 14 days (Figures 8 A and 8E). Next, immunohistochemistry was used to examine the proliferation of SMCs and endothelial cells. It was revealed that the ratio of CD31 positivity in the endothelium of the blood vessels in the IL10-PNP group was significantly higher than that in the IL10-NP group, IL10 group, and saline group, demonstrating that the use of IL10-PNP could promote endothelial cell repair.
a-SMA expression was significantly elevated in the IL10-PNP group (Figures 8C and 8G), in accordance with the more contractile phenotypes in the SMCs. Western blotting results revealed the contractile phenotype-related proteins were significantly decreased after vascular injury, and the application of IL10-PNP significantly reversed the phenotypic transformation of SMCs (Figures 8H and 8I). Systemic application of equal doses of IL10 did not achieve effective plasma concentration and hence did not inhibit restenosis effectively.

**Biocompatibility of IL10-PNP evaluation in vivo**

There were no integrity damage or distinguishable injuries in major organs observed from H&E-stained sections. Meanwhile, complete blood count, biochemical and coagulation parameters in the treatment group had no significant difference from the sham group (Figure S5), indicating the biosafety of IL10-PNP.

**DISCUSSION**

In a previous study, docetaxel-loaded PNPs were fabricated and applied to reduce vascular restenosis (Hu et al., 2015). Docetaxel was the main component of the drug-coated balloon, and its main function was to
Figure 8. Anti-restenosis effect of IL10-PNP in vivo
(A) H&E staining images in the sham group and injured carotid artery with different treatments.
(B) Immunohistochemistry images of CD31 in the sham group and injured carotid artery with different treatments.
(C) Immunohistochemistry images of α-SMA in the sham group and injured carotid artery with different treatments.
(D) Quantitative analysis of intima area and (E) ratio of intima to media in different groups.
(F) The ratio of CD31+ cells on the intimal side in different groups.
inhibit cell proliferation. Hence, it will inhibit endothelial repair while inhibiting smooth muscle cell proliferation. Meanwhile, platelet membrane glycoproteins on PNPs have immunomodulatory effects and will promote the inflammatory response of macrophages after binding to ligands on the surface of macrophages (Carestia et al., 2019; Dann et al., 2018), hence, there would be an increased risk of localized M1 macrophage polarization. These might cause "atherosclerotic-like" restenosis in the long term (Zhang and Chen, 2020). In our study, IL10 was used as the agent to counteract restenosis. The mechanism was the binding effect of platelets to macrophages, which could achieve the targeted delivery of IL10 and stimulate M2 macrophage polarization.

M2 macrophages are also heterogeneous and can be further subdivided into M2a (activated by exposure to IL-4 or IL-13); M2b (induced by immune complexes in the presence of a Toll-like receptor ligand); M2c (elicited with IL-10, glucocorticoids or TGF-β); and M2d (stimulated by TLR agonists through the adenosine receptor) (Shapouri-Moghaddam et al., 2018). Among them, M2c could regulate the local inflammatory response and promote tissue repair (Gensel and Zhang, 2015). Among the various subtypes of M2 macrophages, M2c induced by IL10 has a strong ability to promote tissue repair (Gensel and Zhang, 2015). In this study, we used IL10 to stimulate M2c macrophage polarization, which was characterized by CD163 expression (Raimondo and Mooney, 2018). It proved to have effectively promoted endothelial repair both in vivo and in vitro, reduced subsequent infiltration of immune cells and suppressed local inflammatory responses.

SMCs are different from cardiac or skeletal myocytes because they do not terminally differentiate but retain a high degree of plasticity, which allows SMCs to dramatically alter the phenotype in response to environmental cues and extracellular signals. This property allows SMCs to contribute to the growth, remodeling, and repair of the vasculature, but can also drive vascular restenosis (Chakraborty et al., 2021). Restenosis vessels are mainly composed of SMCs that proliferate and migrate actively. After vascular injury, owing to the stimulation of inflammatory factors, SMCs will change from the contractile phenotype with inactive proliferation to the secretory phenotype with active proliferation and migration, hence leading to restenosis, while M2 macrophages stimulated by IL10 in this study will inhibit the secretory phenotype transformation of smooth muscle cell (Yan et al., 2020). In this study, α-SMA, SM22, and calponin1 were specified as hallmarks of contractile SMC. α-SMA is located at contractile filaments and is essential for the contractile function of smooth muscle cells, while SM22 and calponin1 regulate the contractile function of SMCs. These markers would decrease when SMCs dedifferentiate into secretory phenotype (Furmanik et al., 2019; Owens, 1995; Rensen et al., 2007). OPN is a hallmark of the secretory phenotype of SMCs, which plays a major role in SMCs migration (Jalvy et al., 2007; Zhang et al., 2014b). In our in vitro and in vivo studies, stimulation of macrophages with IL10-PNP effectively reduced the secretory phenotype transformation of SMCs and thus effectively inhibited the occurrence of restenosis.

In previous studies, agents coated by cell membranes were often drugs such as rapamycin and paclitaxel (Hu et al., 2015; Song et al., 2019). These drugs require a certain dose to be effective, so each dose requires a membrane of \(1 \times 10^9\) platelets to coat (Hu et al., 2015; Song et al., 2019), which is close to all of the platelets in the total circulating blood volume of a rat. In this study, IL10 was used as the loading agent. After M2 macrophage polarization caused by IL10, M2 macrophages would also secrete IL10 (Shapouri-Moghaddam et al., 2018), which would cause a local “immune cascade,” leading to more M2 macrophage polarization, so only a small amount of IL10 was needed to be effective. In this study, each dose required membranes of \(10^9\) platelets, close to 10% of platelets in the total circulating blood volume of a rat. From the perspective of translational medicine, it is more feasible to reserve 10% of the total circulating blood volume for cell membrane coating in clinical practice. For the pharmacokinetics of PNP in the present study (Figure S3), the systemic circulation lifetime was slightly longer than previously reported (Hu et al., 2015; Song et al., 2019), possibly owing to the small amount of PNP used in this study. The elimination half-life was similar to NP controls and longer than free IL10 as previously reported (Kim et al., 2020).

Interleukin nanoparticles such as IL4-NP and IL10-NP with modified nanoparticles have been previously fabricated for targeted suppression of inflammation (Kamaly et al., 2016; Raimondo and Mooney, 2018). Interleukin 10 is a 35 kD dimer consisting of two monomers bonded by a noncovalent bond. Consequently,
the protein structures may undergo rupture during preparation and lose their biological activity. A previous study added D-(+)-glucosamine hydrochloride as an IL-10-stabilizing additive and cryoprotectant during the preparation process of IL10-NP and selected PLGA as a nanoparticle carrier (Kamaly et al., 2016). The biological activity of IL10 was protected during the preparation and had good loading efficiency. In this study, a similar IL10 preparation method was used before encapsulation with platelet cell membranes. The targeting capability was significantly improved, as well as the ability to inhibit restenosis. In vitro experiments verified that IL10-PNP has satisfactory loading and release ability (Figure 1) and maintains the good biological activity of IL10. In vitro, IL10-PNP, IL10-NP, and IL10 had similar effects on regulating the polarization of M2 macrophages, inhibiting the proliferation and secretory phenotypic transformation of SMCs (Figures 4B and 4C) and promoting endothelial cell repair (Figure 5). However, owing to the targeted delivery effect of IL10-PNP, its biological function is stronger in vivo and has a better inhibitory effect on restenosis (Figure 8). Further work is still required to improve the present PNP. From in vivo studies, it was observed that a large proportion of PNP was metabolized in the liver and spleen. Previous studies have emphasized the role of membrane surface modification in improving PNP performance (Ai et al., 2021). In the future, PNP could be improved by cell membrane modification, to reduce the deposition in the liver and spleen. IL10 has been confirmed to regulate macrophage reprogramming through various mechanisms in a previous study (Ip et al., 2017). IL10-PNP can be used as a tool to study the mechanism of IL10 regulating macrophages in vivo owing to its targeted delivery characteristics.

Limitations of the study
In the present study, IL10-PNP can effectively target and regulate the M2 macrophage polarization at the site of vascular injury to reduce restenosis. Owing to the properties of platelet membrane proteins, a large proportion of PNP was metabolized in the liver and spleen. In future studies, we hope to modify platelet membrane protein structure to reduce the destruction in the liver and spleen and enhance targeting ability.

CONSENT FOR PUBLICATION
All authors gave their consent for publication.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
For the in vitro experiment, platelets were collected from healthy volunteers (healthy university students) without any anti-platelet drugs taken for at least 14 days, and informed consent was obtained. All animal studies were conducted in accordance with the Animal Management Rules of the Chinese Ministry of Health, and the Animal Ethics Committee of Peking Union Medical College reviewed and approved the procedures of the studies (No. XHDW-2019-001).

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Date and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Materials, cell lines, and animals
- **METHOD DETAILS**
  - Preparation and characterization of IL10-NP and IL10-PNP
  - Protein expression of PNP
  - Induction and identification of macrophages
  - Cell culture & binding effect of IL10-PNP on macrophage
  - Coculture system
  - Regulation role of IL10-PNP on macrophage in vitro
  - Cell viability assay
  - Real-time quantitative polymerase chain reaction (RT-qPCR) analysis & Western blot
  - Transwell assay
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105147.

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AUTHOR CONTRIBUTIONS

Fengshi Li: Conceptualization, Formal analysis, Methodology, Investigation, Writing - Original Draft; Zhihua Rong: Methodology, Validation, Formal analysis; Rui Zhang: Validation, Visualization; Shuai Niu: Methodology, Investigation; Xiao Di: Writing - Review & Editing; Leng Ni: Writing - Review & Editing, Supervision; Changwei Liu: Conceptualization, Supervision, Funding acquisition. All authors approved of the article.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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### STAR★METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| anti-P-selectin antibody | ABclonal | CAT#A4989; RRID: AB_2863406 |
| anti-CD42b antibody | Invitrogen | CAT# PAS-86799; RRID: AB_2803559 |
| anti-CD40 L antibody | Proteintech | CAT# 16668-1-AP; RRID: AB_2076318 |
| anti-CD61 antibody | Biolegend | CAT# 336402; RRID: AB_1227584 |
| anti-CD86 antibody | Biolegend | CAT# 374205; RRID: AB_2721632 |
| anti-CD163 antibody | Biolegend | CAT# 333609; RRID: AB_2291272 |
| anti-F4/80 antibody | Abcam | CAT# ab16911; RRID: AB_443548 |
| anti-CD86 antibody | Abcam | CAT# ab238468 |
| anti-CD163 antibody | Abcam | CAT# ab182422; RRID: AB_2753196 |
| anti-CD31 antibody | Abcam | CAT# ab281583 |
| anti-α-SMA antibody | CST | CAT# 19245; RRID: AB_2734735 |
| anti-α-SMA antibody | Proteintech | CAT# 14395-1-AP; RRID: AB_2223009 |
| anti-SM-22 antibody | Abcam | CAT# ab14106; RRID: AB_443021 |
| anti-β-actin antibody | Proteintech | CAT# 66009-1-lg; RRID: AB_2687938 |
| anti-calponin1 antibody | CST | CAT# 17819; RRID: AB_2798789 |
| anti-CD11b antibody | Abcellonl | CAT# A1581; RRID: AB_2763232 |
| **Chemicals, peptides, and recombinant proteins** | | |
| PLGA | Sigma-Aldrich | CAT# P2191 |
| PVA | Sigma-Aldrich | CAT# 363170 |
| Rat recombinant IL10 | Abcam | CAT# ab256036 |
| Human recombinant IL10 | Abcam | CAT# ab259402 |
| Lipopolysaccharides from Escherichia coli O111:B4 (LPS) | Sigma-Aldrich | Cat# L3024 |
| **Critical commercial assays** | | |
| Rat IL10 ELISA kit | Solarbio | CAT# SEKR-0006 |
| Human TNF-α ELISA kit | Abcam | CAT# ab181421 |
| Human IL-1β ELISA kit | Abcam | CAT# ab214025 |
| Human IL-6 ELISA kit | Abcam | CAT# ab178013 |
| TRizol | Ambion | CAT# 15596026 |
| PrimeScript™ RT reagent Kit | Takara | CAT# RR047A |
| SYBR green mix | Thermo Fisher | CAT# A25776 |
| **Experimental models: Cell lines** | | |
| Human: Cell line THP-1 | ATCC | CAT#TIB-202 |
| Human: Primary Smooth muscle cell | ScienceCell | CAT# 6110 |
| **Experimental models: Organisms/strains** | | |
| Sprague-Dawley Rats | Charles River Laboratory | N/A |
| **Software and algorithms** | | |
| GraphPad Prism version 7.0 | GraphPad Software | N/A |
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Liu Changwei (liucw@vip.sina.com).

**Materials availability**
This study did not generate new unique reagents.

**Date and code availability**
All data are available in the paper and in supplemental information, and/or from the corresponding author upon reasonable request.

This paper does not report original codes.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Materials, cell lines, and animals**
PLGA and poly (vinyl alcohol) (PVA) were purchased from Sigma-Aldrich (USA). THP-1-cell lines were purchased from ATCC (USA). All animal studies were conducted in accordance with the Animal Management Rules of the Chinese Ministry of Health, and the Animal Ethics Committee of Peking Union Medical College reviewed and approved the procedures of the studies (No. XHDW-2019-001). Adult male Sprague-Dawley rats (200–250 g; 8- to 12-week-old) were obtained from Charles River Laboratory (Beijing, China) and housed in the Laboratory Animal Center of Peking Union Medical College Hospital. Rats were housed in a climate-controlled facility (25–28°C, humidity 50%–60%, 12 h light/dark cycle) and had free access to food and water.

**METHOD DETAILS**

**Preparation and characterization of IL10-NP and IL10-PNP**
Platelets were collected from platelet-rich plasma (PRP) as previously described (Hu et al., 2015). BioTek Synergy H1 plate reader was used to evaluate platelet aggregation. The absorbance at 650 nm was measured, and 0% platelet aggregation was defined as the OD650 value platelet dispersed in calcium-free Tyrode’s buffer. The sample’s OD650 value was measured 30 minutes incubating after activation by ADP with different volume ratios of ACD added. Platelet aggregation rate was defined as the ratio of the sample’s OD650 value to the 0% OD650 value. Platelet aggregation was significantly decreased with 1/4 volume of ACD added, and there was no significant difference between 1/4 volume, 1/3 volume, and 1/2 volume (Figure S1). Then, platelets were activated by 1 mmol/L adenosine 5’-diphosphate (ADP; Sigma-Aldrich, USA) with 1/4 volume of acid citrate dextrose solution (ACD; Sigma-Aldrich, USA) added to prevent platelet aggregation. A repeated freeze-thaw process was performed to extract the platelet membrane (Hu et al., 2015). IL10-NPs were prepared by a modified double solvent evaporation method as previously described with slight modification (Zeng et al., 2019). In brief, 1 mL of PLGA (Sigma-Aldrich, USA) solution in ethyl acetate (60 mg/mL) was mixed with recombinant rat IL10 (Abcam, Cambridge, UK) and D- (+)-glucosamine (4 and 6 w/w % of total PLGA mass) dissolved in 100 µL ultrapure DNase/RNase-free distilled water. The solution was made into the initial W1/O (water/oil) emulsion after sonication for 1 min. Next, 5 mL of 1% PVA (Sigma-Aldrich, USA) aqueous solution was added to the resulting emulsion. Two minutes of sonication was performed to obtain the final W1/O/W2 emulsion. The final emulsion was injected into 50 mL of 0.3% (w/v) PVA solution and evaporated overnight under stirring to remove ethyl acetate. The resulting IL10-NPs were washed 5 times in ultrapure DNase/RNase-free distilled water by centrifugation at 13,000 rpm for 5 minutes each time and resuspended in PBS, pH 7.4. For IL10-PNP preparation, IL10-NPs loaded with 1 µg of IL10 were mixed with 1.5 mL of platelet membrane (containing approximately 3×10^9 platelets), then sonicated at a power of 100 W for 2 minutes (Hu et al., 2015). For targeting effect evaluation, IL10 in nanoparticles was replaced by 5 µg of 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindotri-carbocyanine iodide (DiR, Invitrogen), and DiR-loaded NPs (DiR-NPs) and DiR-PNPs were prepared in the same way. For the in vitro experiment, platelets were collected from healthy
volunteers (healthy university students) without any anti-platelet drugs taken for at least 14 days, and informed consent was obtained. Recombinant human IL10 (Abcam, USA, ab214119) was used for IL10-NP and IL10-PNP.

The morphology of IL10-NPs and IL10-PNPs were examined by a transmission electron microscope (TEM; JEOL JEM2100F, JEOL Ltd. Japan) after staining with phosphotungstic acid. An evaluation of the encapsulation efficacy and loading capacity was conducted as previously described (Zeng et al., 2019). Disperse 10 mg of IL10-NP/IL10-PNP in 5 mL of 2.5% SDS/0.04 M NaOH. Hydrolysis of the nanoparticles was carried out in an orbital shaker at 37°C and 100 rpm. The IL10 concentration was measured by a rat IL10 enzyme-linked immunosorbent assay (ELISA) kit (Solarbio, China, SEKR-0006) according to the manufacturer’s instructions. Based on the ratio of loaded to initial IL10, we calculated the IL10 encapsulation efficiency, and the loading capacity was calculated based on the ratio of loaded to total IL10. The size and zeta potential of IL10-NPs and IL10-PNPs were measured by dynamic light scattering (DLS) in deionized water using a Zetasizer Nano S (Malvern Instruments, UK). The amount of released IL10 at each time point was measured by a Rat IL10 ELISA kit (Solarbio, China, SEKR-0006).

**Protein expression of PNP**

Key glycoproteins that bind to macrophages on the surface of platelets were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting. Briefly, lyse platelet membrane vesicles and empty PNP with radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor (1:100). Total protein contents were quantified by a BCA Protein Assay Kit (Thermo Fisher Scientific, USA, 23225), then mix samples with SDS–PAGE loading buffer and heat for 10 min at 100°C. Load samples with equal amounts of protein (30 μg/well) on 10% SDS–PAGE gels and run at 120 V for 2 hrs. Stain the gel with Coomassie Blue for 2 hrs and wash overnight. The gel was also transferred to polyvinylidene difluoride membranes. Identification of key proteins by Western blotting was probed by primary antibodies, including anti-P-selectin antibody (ABclonal, China, A4989), anti-CD42b antibody (Invitrogen, USA, PA5-86799), and anti-CD40 L antibody (Proteintech, China, 16668-1-AP), at 4°C overnight and incubated with respective secondary antibodies.

**Induction and identification of macrophages**

Macrophages were derived from THP-1-cell lines (ATCC, USA, TIB-202), after 24 hours of stimulation by PMA (10 ng/mL), macrophages would adhere to the wall of a T25 flask, then, cells were seeded to 24 wells plate and fixed the cells with 4% paraformaldehyde for 15 min. After washing in PBS containing 10% goat serum, the cells were incubated overnight with anti-CD11b antibody (ABclonal, China, A1581) at 4°C, then incubated with the appropriate secondary antibodies and imaged by a confocal laser scanning microscope (Figure S2).

**Cell culture & binding effect of IL10-PNP on macrophage**

Macrophages were derived from THP-1-cell lines (ATCC, USA, TIB-202) as previously described (Baxter et al., 2020) (Figure S2), and platelets were collected from healthy volunteers. For the binding function of IL10-PNP to macrophages in vitro, IL10-PNP (containing 0.1 μg IL10) and macrophages (2 × 10⁶ cells/well) were seeded on glass coverslips placed in 24-well plates. As controls, preactive platelets and macrophages were cultured together. Macrophages were cultured alone as a blank group. After 2, 12, and 24 hours of culture, removed the supernatant and fixed the cells with 4% paraformaldehyde for 15 min. After washing in PBS containing 10% goat serum, the cells were incubated overnight with anti-CD61 antibody (Biolegend, USA, A1581) at 4°C, then incubated with the appropriate secondary antibodies, and imaged by a confocal laser scanning microscope.

To evaluate the binding ratio of IL10-PNP to macrophages, after 2, 12, and 24 hours of culture, the supernatant was removed and cells were harvested and stained with anti-CD61 antibody (Biolegend, USA, 336402) at 4°C. Then the coverslips were washed with PBS, incubated with the appropriate secondary antibodies, and imaged by a confocal laser scanning microscope.

**Coculture system**

To mimic the inflammatory environment after vascular injury, a co-culture system with platelet-stimulated macrophage polarization was compared with classical LPS-stimulated (100 ng/mL) macrophage activity.
polarization. The results showed that there was no significant difference in the proportion of M1 polarization between platelet-stimulated macrophages and the LPS group (Figure S4).

To assess the regulatory role of IL10-PNP on macrophages in vitro, a coculture system of IL10-PNP (containing 0.1 µg IL10) with macrophages (2 × 10^6 cells/well) and platelets (2 × 10^8 cells/well) was established. IL10-PNP was replaced by IL10-NP (containing 0.1 µg IL10) or IL10 (0.1 µg) or PBS for the control. Macrophages were cultured alone as a blank group. Empty PNP equal to IL10-PNP above was added to macrophages to access the regulation of macrophage inflammation by empty PNP (Figure S4).

Regulation role of IL10-PNP on macrophage in vitro
After 24 hours of coculture, the cells were digested and stained with anti-CD86 antibody (Biolegend, USA, 374205) to identify M1 macrophages and anti-CD163 antibody (Biolegend, USA, 333609) to identify M2 macrophages. Then, flow cytometric analysis was performed on an LSRFortessa™ (BD Biosciences, USA). The chemokines in the supernatant including TNF-α, IL-1β, and IL-6 secreted by M1 macrophages were measured using the corresponding ELISA kits (Abcam, USA) according to the manufacturer’s instructions.

Cell viability assay
To evaluate the functional changes of smooth muscle cells (SMCs) after IL10-PNP application, SMCs (ScienCell, 6110) were plated on the bottom chamber of a Transwell (Corning Incorporated, USA) (1 × 10^6 cells/well), and the coculture system mentioned above, including IL10-PNP, platelets, and macrophages, was seeded in the upper chamber. As a control, IL10-PNP was replaced by IL10-NP or IL10, or PBS. SMCs were cultured alone as a blank group. The proliferation function of SMCs was assessed by Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Japan) following the manufacturer’s instructions. After 1–5 days, the SMCs were harvested and dispensed in a 96-well plate and added 10 µL/well CCK-8 reagent followed by incubating at 37°C for 2 h. Then, the absorbance was measured at 450 nm by a microplate spectrophotometer (Varioskan, Thermo Fisher, USA). Edu assay was applied to detect the proliferation function of SMCs, after 24 hours of coculture, Ethynyl-deoxyuridine assay (RiboBio, China) was used followed by manufacturer’s instructions.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis & Western blot
To determine phenotypic transformation of SMCs after IL10-PNP application, gene expression levels of osteopontin (OPN) and SM22 which correlated with secretory phenotype and contraction phenotype respectively were detected by RT-qPCR. After 24 hours of coculture, the total RNA of SMCs was isolated by the TRIzol Reagent (Ambion, USA) and 1 µg RNA was reverse transcribed by the reverse transcription kit (Takara, Japan). β-actin was applied as an internal control for RNA-related experiments. RT-qPCR was performed using SYBR green mix (Thermo Fisher Scientific, USA) on QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, USA). The forward (F) and reverse (R) primers are presented as followed (5′-3′): OPN-F: CAGCCCGTGGGAAGGACAGTT-ATG; OPN-R: TCACATCGGAATGCTCATTGCTCTC; SM22-F: GCCAGTCCAAAATCGAAGAAG; SM22-R: CAGAA TCACGCCATTCTTCAG; β-actin-F: TGACGTGGACATCCG-CAAAG; β-actin-R: CTGGAAGGTGGACAGGAGG. The relative expression of target genes was calculated by the 2^(-ΔΔCt) method. Gene expressions were normalized to β-actin.

And Western blot as described above was performed to detect the contractile phenotype-related proteins of SMCs probed with corresponding primary antibodies, include anti-α-SMA (Proteintech, China, 14395-1-AP), anti-SM-22(Abcam, USA, ab14106), anti-β-actin (Proteintech, China, 66009-1-lg), anti-calponin1 (CST, USA, 17819).

Transwell assay
The migration function of smooth muscle cells was assessed by a transwell assay. The coculture system mentioned above was seeded in the lower chamber, and smooth muscle cells were seeded in the upper transwell chamber. After 24 hours of incubation at 37°C and 5% CO2, the filter was removed, and cells in the upper chamber were removed with a cotton tip. Smooth muscle cells that migrated into the lower chamber were fixed with 4% paraformaldehyde, and 1% crystal violet in 2% ethanol was used for cell staining.
**Wound healing assay & tube formation assay**

For the function of IL10-PNP to promote endothelial cell repair, wound healing assay and tube formation assay were conducted. Plate human umbilical vein endothelial cells (HUVECs) into a 24-well plate and culture for 24 h to reach a fusion rate of 80%. The cells were then scratched with a 200-µL sterile pipette tip, and the coculture systems mentioned above, including IL10-PNP, platelets, and macrophages, were seeded in a Transwell chamber and placed on a 24-well plate. As a control, IL10-PNP was replaced by IL10-NP or IL10, or PBS. HUVECs were cultured alone as a blank group. The cells were cocultured for 24 hours, and the recorded images were recorded at 0 and 24 hours.

After 24 hours of coculture, HUVEC were harvested and added in 96-wells plate coated with Matrigel (Corning Incorporated, USA) and added medium from the blank group and co-culture system (PBS group, IL10 group, IL10-NP group, IL10-PNP group), respectively. Imaged after incubated for 6 hours.

**Model of vascular injury by angioplasty**

All rats underwent balloon-induced carotid artery injury as previously described. Briefly, after anesthetization with pentobarbital sodium (40 mg/kg, i.p.), an incision in the midline of the neck was made, and the left common carotid artery and carotid bifurcation were exposed by blunt dissection. A temporary clamp was placed proximal to the left common carotid artery, inner carotid artery, and external carotid artery. An arteriotomy was made on the external carotid artery and the 2F Fogarty arterial embolectomy catheter (Edwards Lifesciences, USA) was slowly inducted into the common carotid artery, and the catheter was slowly inflated to a determined pressure (2 atm) and withdrawn with rotation 3 times. The wound was then closed with 4-0 sutures.

**The circulation retention property of PNP**

DiR-labeled PNPs were applied to study the blood clearance of PNP. At different timed intervals, 100 µL blood was collected in 96 wells plates, and the fluorescence was detected by IVIS Lumina XRMS Series III (PerkinElmer, USA). Values were normalized based on the value of 2 minutes (Figure S3).

**Ex-vivo near-infrared fluorescence imaging**

To detect the targeting effect of PNP on vascular injury, rats were randomly divided into three groups after wound closure: the control group was injected intravenously with DiR saline solution, and the NP group was injected intravenously with DiR-labeled NPs, and the PNP group was injected intravenously with DiR-labeled PNPs. All rats received 5 µg DiR injections via the tail vein. The rats were sacrificed 24 hours after the procedure (Figure S3), and the fluorescence intensities of the bilateral carotid arteries, heart, liver, spleen, lung, and kidney were measured by IVIS Lumina XRMS Series III (PerkinElmer, USA).

**Release function of IL10-PNP in vivo**

To exam, the release function of IL10-PNP in vivo, the level of IL10 in different organs was detected. 24 hours after treatment, frozen tissues (carotid artery, heart, liver, spleen, lung, kidney) were homogenized in cell lysis buffer with proteases added. After centrifuged, the BCA Protein Assay Kit (Thermo Fisher Scientific, USA, 23225) was used to adjust the supernatants to the same protein concentration and the IL10 concentration was measured by a rat IL10 ELISA kit (Solarbio, China, SEKR-0006) according to the manufacturer’s instruction.

**Histology and immunohistologic analysis**

To verify IL10-PNP function, rats were randomly divided into three groups after wound closure. The saline group was injected intravenously with saline, the IL10 group was injected intravenously with 1 µg IL10, the IL10-NP group was injected intravenously with IL10-NP with 1 µg IL10 loaded, and the IL10-PNP group was injected intravenously with 1.5 mL of IL10-PNPs with 1 µg of IL10 loaded on Day 0 and Day 5. On Day 14, the rats were euthanized with an overdose of a mixture of ketamine and xylazine and then perfused with PBS and 4% paraformaldehyde (PFA) at a pressure of 125–145 mm Hg. Segments of the left and right carotid arteries were fixed with 10% formalin, embedded in paraffin, and cut into 5 µm sections. Slides were stained with hematoxylin and eosin (H&E) to evaluate areas of intima and media. For immunofluorescence, sections were incubated with antibodies directed against F4/80 (Abcam, USA, ab16911) to locate macrophages, CD86 (Abcam, USA, ab238468) to locate M1 macrophages and CD163 (Abcam, USA, 182422) to locate M2 macrophages. For immunohistochemistry, sections were incubated with CD31 (Abcam, USA,
ab281583) to identify endothelial cells and α-SMA (Cell Signaling Technology, USA, 19245) to identify contractile SMCs, which are a phenotype with inactive proliferation and secretion functions.

Frozen left carotid artery tissues were homogenized in cell lysis buffer with proteases added. After centrifuged, the BCA Protein Assay Kit (Thermo Fisher Scientific, USA, 23225) was used to adjust the supernatants to the same protein concentration, and Western blot as described above was performed to detect the contractile phenotype-related proteins of SMCs probed with corresponding primary antibody, include anti-α-SMA (Proteintech, China, 14395-1-AP), anti-SM-22(Abcam, USA, ab14106), anti-β-actin (Proteintech, China, 66009-1-Ig), anti-calponin1 (CST, USA, 17819).

Biocompatibility evaluation
Blood samples were obtained 24 hours after treatment, and complete blood count, and biochemical and coagulation parameters were measured. And major organs (heart, liver, spleen, lung, kidney) were harvested and stained with H&E 14 days after treatment (Figure S5).

QUANTIFICATION AND STATISTICAL ANALYSIS
All data were shown as the means value with standard deviation. Statistical analyses were performed by GraphPad Prism version 7.0 (GraphPad Software, USA). The differences between the two groups were analyzed by the Student’s t-test or non-parametric test. One-way analysis of variance (ANOVA) with Tukey’s post-hoc test was used to compare multiple groups. Values of p less than 0.05 were considered statistically significant.