Immunomodulatory hybrid micro-nanofiber scaffolds enhance vascular regeneration

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A B S T R A C T
The inertness of synthetic polymer materials and the insufficient mechanical strength of reprocessed decellularized extracellular matrix (dECM) limited their promotive efforts on tissue regeneration. Here, we prepared a hybrid scaffold composed of PCL microfibers and human placental extracellular matrix (pECM) nanofibers by co-electrospinning, which was grafted with heparin and further absorbed with IL-4. The hybrid scaffold with improved hemocompatibility firstly switched macrophages to anti-inflammatory phenotype (increased by 18.1%) and then promoted migration, NO production, tube formation of endothelial cells (ECs), and migration and maturation of vascular smooth muscle cells (VSMCs), and ECM deposition in vitro and in vivo. ECs coverage rate increased by 8.6% and the thickness of the smooth muscle layer was 1.8 times more than PCL grafts at 12 weeks. Our study realized the complementary advantages of synthetic polymer materials and dECM materials, and opened intriguing perspectives for the design and construction of small-diameter vascular grafts (SDVGs) and immune-regulated materials for other tissue regeneration.

1. Introduction
Small-diameter vascular grafts (SDVGs, inner diameter <6 mm) are urgently needed to treat various cardiovascular diseases, such as coronary artery bypass grafting, peripheral vascular replacement, and hemodialysis arteriovenous fistula [1,2]. At present, synthetic SDVGs made of expanded polytetrafluoroethylene (ePTFE), polyethylene terephthalate (Dacron) and other commercially available synthetic polymers showed unsatisfactorily low patency rates, due to infection, acute thrombosis, and intimal hyperplasia (IH) [3]. Compared with non-degradable polymers, SDVGs constructed from degradable polymers (e.g., polycaprolactone (PCL), poly (l-lactide-co-e-caprolactone) (PLCL), poly (glycerol sebacate) (PGS), etc.) have high biocompatibility, mechanical properties, processability and controllable degradation performance, which improved the regenerative capacity of grafts [4–6]. However, those grafts’ tissue regeneration efforts and patency rate are still inferior to autografts. The issues mentioned above are attributable to the biological inertness and lack of reaction sites for grafting active substances in synthetic materials, making the scaffolds inefficient for anti-coagulant and pro-regenerative modification [7–9].

As an ultimate bioactive material, the decellularized extracellular matrix (dECM) possessed exceptional biocompatibility and bioactivity [10,11] and abundant reaction sites for functional modification. Compared with common allogeneic or xenogeneic dECM materials, human placental extracellular matrix (pECM) possess a wide range of sources, convenient access, good biocompatibility, few ethical issues,
2.1. Materials

which further promoted migration, NO generation and tube formation of activity modification of hybrid scaffolds for SDVG construction. Schoen et al. reported the preparation of porcine myocardial dECM nanofiber scaffolds by electrospinning for the first time. They demonstrated that the scaffolds retained components and microstructure as cardiac ECM, and had attractive biocompatibility in vitro and in vivo [14]. Smoak et al. further used electrospinning to fabricate a rabbit skeletal muscle dECM nanofiber scaffolds with tunable physicochemical properties to control mouse myoblast growth and myotube formation [15]. However, the above electrospinning scaffolds are challenging to apply to in situ regeneration of vascular tissue due to insufficient mechanics. The composite of dECM nanofibers and synthetic polymer materials is expected to meet implants’ mechanical and bioactivity requirements.

Host immune response to SDVGs is crucial in influencing materials-induced tissue remodeling [16,17]. Macrophages play essential roles in both host defense and tissue repair [18]. Macrophages can be polarized into pro-inflammatory and pro-healing subtypes. Increasing evidence suggested anti-inflammatory macrophages played an active role in the vascular tissue microenvironment, promoting viability and proliferation of endothelial cells (ECs) and migration and differentiation of vascular smooth muscle cells (VSMCs) [19]. IL-4 is a typical cytokine and can switch macrophages into an anti-inflammatory phenotype. Nathan et al. reported that the failure of coronary artery bypass grafting (CABG) was associated with the lack of IL-4 [20]. Richard et al. found that artificial grafts coating with IL-4 inhibited fibrous encapsulation and IH [21]. In addition, IL-4 plays a positive role in regenerating nerves, muscles, and other tissues [22,23]. Furthermore, good hemocompatibility is essential to reduce platelet adhesion, avoid thrombosis and enhance vascular patency. Heparin has been used as a typical anti-coagulant in clinical applications [24]. As a negatively charged glycosaminoglycan (GAG), it has strong electrostatic interactions with growth factors and can achieve sustained release of IL-4 [25].

Based on the above aspects, the co-electrospinning technique was used to fabricate hybrid SDVGs composed of PCL microfibers and pECM nanofibers (PCL-pECM). PCL microfibers enhanced mechanical properties and improved cell migration, while pECM nanofibers provided biological activity and active modification sites for heparinization (PCL-pECM/H) and further IL-4 loading (PCL-pECM/H-IL-4). The hybrid scaffolds exhibited improved hemocompatibility and cytoocompatibility, and could facilitate the anti-inflammatory polarization of macrophages, which further promoted migration, NO generation and tube formation of ECs as well as migration and maturation of VSMCs in vitro. Meanwhile, the hybrid scaffolds promoted the endothelialization, smooth muscle regeneration and ECM deposition in the rat’s abdominal artery defects model. Our study provides sufficient experimental evidence and new design ideas for the mechanical enhancement, structural regulation, and activity modification of hybrid scaffolds for SDVG construction.

2. Materials and methods

2.1. Materials

Poly (ε-caprolactone) (PCL) pellets (Mn 80,000) and Polyethylene oxide (PEO) powder (Mn 600,000) were purchased from Sigma (St Louis, USA). Hexafluoroisopropanol (HFIP) was purchased from Aladdin (Shanghai, China). Methanol, alcohol, acetone and xylene were obtained from Tianjin Chemical Reagent Company (Tianjin, China). TissueTek OCT compound was obtained from Sakura Finetek (Torrance, CA, USA). Deoxyribonuclease I (DNase I) and ribonuclease (RNase) were obtained from Sigma-Aldrich (St. Louis, Missouri, US). Sodium Dodecyl Sulfate (SDS) was bought from Alfa Aesar (London, UK). Sprague-Dawley (SD) rats (male, weight 280–320 g) were purchased from SPF (Beijing) Biotechnology Co., Ltd. (Beijing, China). All animal experiments were approved by the Animal Experiments Ethical Committee of Nankai University and complied with the Guideline for Care and Use of Laboratory Animals. The accreditation number of the laboratory is SYXK(Jin) 2019-0001 promulgated by Tianjin Science and Technology Commission.

2.2. Decellularization procedure

Full term human placentas were obtained from healthy donors undergoing delivery section at the Tianjin Central Hospital of Gynecology and Obstetrics. Informed consent was obtained from three donors (female, Asian). All studies using human samples were approved by the Donation Service of Tianjin Central Hospital of Gynecology and Obstetrics and Tianjin Central Hospital of Gynecology and Obstetrics’s clinical research ethics committee (approval number2022KY056), which has regulations consistent with the Helsinki Declaration. The placenta was subsequently frozen at −80 °C and thawed before decellularization, then it was decellularized and lyophilized according to our previously published work [26]. Then pECM was frozen in liquid nitrogen, and crushed into powder in a cryogenic tissue grinder (Shanghai Jingxin Industrial Development Co., Ltd. China).

2.3. Characterization of native placenta, pECM and epECM matrix

The remaining DNA content in the native placenta and pECM was quantified using a Quanti-IT PicoGreen Assay (Invitrogen, Grand Island, NY). Specifically, DNA were extracted from native placenta and pECM prior to quantification, according to the manufacturer’s instructions. After diluting the DNA of the sample and the fluorescent dye, add 125 µL of the gradient DNA standard solution and the test solution to the 96-well plate, then add 125 µL of the diluted DNA fluorescent dye, avoid light and react for 5 min. A multifunctional microplate reader (excitation wavelength: 480 nm, emission wavelength: 520 nm) was used to measure the fluorescence intensity of the sample and quantified by a standard curve of DNA. GAGs content was measured using a ELISA kit (Shanghai Enzyme-linked Biotechnology, China). Briefly, homogenate PBS (pH 7.4) was added to samples of native placenta, pECM and epECM powder with the mass-to-volume ratio of 1:9. Then a homogenizer was used to fully homogenize the tissue for 30 min, and the supernatant was carefully collected after centrifuge (3000). The absorbance was measured at 450 nm and quantified by a standard curve of GAGs. Collagen content was measured using hydroxyproline assay kit (Solarbio, China) according to the manufacturer’s instructions. Briefly, native placenta, pECM and epECM powder were hydrolyzed with 12 M hydrochloric acid for 3 h at 110 °C. The acid-hydrolyzed samples were further dried under vacuum evaporation at 80 °C for 40 min. Then chloramine T mixture was added to convert the hydroxyproline to a pyrrole. Finally, Ehrlichs Reagent was added to the solution to react with the pyrroline and produce a chromophore. The absorbance of the samples was measured at 540 nm and quantified by a standard curve of hydroxyproline.

2.4. Placental proteomics identification

The samples were digested by trypsin (1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h digestion) and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to obtain polypeptides. The peptide mixture was separated by high-pressure liquid chromatography (HPLC), and then electrospray was performed on an ion trap mass spectrometer. The mass tolerance for precursor ions was set as 20 ppm in the First search and 5 ppm in the Main search, and the mass tolerance for fragment ions was set as 0.02 Da. These peptides are further broken by
Peptides in UniProt database were analyzed and identified by Proteome Discoverer software (Thermo scientific). If a protein was not identified in the database, the basic local alignment search tool (BLAST) was used to find regions of local similarity between sequences in the UniProt database to identify members of the same gene family.

2.5. Fabrication of grafts and bioactive modification

The pure PCL scaffolds were fabricated by electrospinning [4]. Briefly, 25% (w/v) PCL was dissolved in CHCl3/MeOH (5:1, v/v), the electrospinning conditions were as followed: needle-collector distance: 15 cm, flow rate: 8 mL/h, voltage: 12 kV, needle size: 21-G. The micro-nano hybrid scaffolds (PCL-epECM) were fabricated by co-electrospinning, the spinning condition of PCL microfiber was the same as the above mentioned. The electrospinning conditions of epECM nanofiber were as followed: 5% (w/v) pECM and 0.1% (w/v) PEO were dissolved in HFIP, needle-collector distance: 10 cm, flow rate: 3 mL/h, voltage: 15 kV, needle size: 21-G. The epECM nanofibers of the hybrid scaffold were heparinized by EDC/NHS. EDC (120 mM) and NHS (60 mM) were added to 40 mL MES buffer (PH 5.5), then 0.04 g heparin was added to the buffer and activated in an ice bath for 2 h. The hybrid scaffold was put into the activated heparin solution and soaked at room temperature for 12 h, then the scaffold was cleaned with PBS to remove the cross-linking agent and PEO. The heparinized scaffold (PCL-epECM/H) was incubated in recombinant human IL-4 (1 μg/mL, Solarbio, China) for 4 h at 37 °C (PCL-epECM/H-IL-4). All grafts were stored at 4 °C until use.

Characterization of vascular grafts: The distribution of different fibers was visualized on a laser scanning confocal microscope (LSCM, Leica TCS SP8, Germany). PCL microfiber and pECM nanofiber were doped with Dil (0.01% w/v) and DiO (0.01% w/v) by physical blending, respectively. The structure and morphology of grafts were observed under a scanning electron microscope (SEM, Hitachi, X-650, Japan) at 1000× magnification. The water contact angles of the different scaffolds (n = 5) were measured using the sessile drop method with a Harke-SPOCA goniometer (Beijing, China). For the biological activity assay, the viability of RAECs was assessed using a Live/Dead assay kit (Yesen, China) according to the manufacturer’s instructions (n = 5). RAECs were seeded at a density of 4 × 10^3 cells/well on different scaffolds (PCL, PCL-epECM/H, or PCL-epECM/H-IL-4). Live and dead cells were stained green and red, respectively, and images were captured by a laser scanning confocal microscope LSCM (Leica TCS SP8, Germany). The cell viability was calculated using Image J and expressed as the proportion of live cells to the total cells. A Cell Counting Kit (CCK)-8 assay was used to assess the cytocompatibility of different scaffolds. RAECs were seeded at a density of 8 × 10^3 cells/well (n = 5) on different scaffolds and VSMCs were seeded at a density of 5 × 10^3 cells/well (n = 5), respectively. At the predetermined time points, CCK-8 dilution in DMEM (1:10) was added to each well after removing the media. After incubation for 1 h at 37 °C, 100 μL supernatant was removed from each well and added to a 96-well plate. The optical density (OD) value at 450 nm was determined using a microplate reader (Bio-Rad, USA) to evaluate the viability of the cells in each well.

2.6. AV-shunt and anticoagulation detection

AV-shunt assay was performed in rats to investigate the blood compatibility of grafts. Rats were anesthetized with 0.15% (v/v) isoflurane gas throughout the procedure. Heparin (100 Units/kg) was administered as an anticoagulant agent. The grafts (PCL, PCL-epECM/H, PCL-epECM/H-IL-4)) were cut into a 1.5 cm and sterilized (n = 3). Then the grafts were connected to the abdominal aorta at one end and to the abdominal vein at the other end (As shown in Fig. 2A), using 24-G indwelling needles to establish extracorporeal circulation, and three grafts were connected in one circulation. After circulation for 1.5 h, the circuit was perfused with physiological saline. Samples were equally divided into two parts. One was fixed in 2.5% glutaraldehyde overnight and dehydrated by a gradient of ethanol for SEM assay, and the other was stained with mepacrine (Sigma, St Louis, USA) for 30 min at 37 °C and then observed under LSCM (Leica TCS SP8, Germany).

2.7. Clotting time assays

The grafts were cut into 5 mm in length for experiment (n = 6). A volume of 500 μL of human plasma was incubated with the grafts for 1 h at 37 °C. After that, the grafts were removed. FIB, PT, TT, and APTT were analyzed by an automatic SYMSMEXCS-5100 coagulation analyzer instrument (Siemens Healthineers, Erlangen, Germany).

2.8. Cell culture

Monocytes derived from rat bone marrow were induced to differentiate into macrophages by granulocyte-macrophage colony stimulating factor (GM-CSF, Wu Han Cloud-clone Co., Ltd., China) for 7 days in 1640 medium (Gibco, Grand Island, NY) supplemented with 15% heat-inactivated fetal bovine serum (FBS, Gibco, Australia). The RAECs were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. The RAECs were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% FBS, and 100 U/mL penicillin and 100 U/mL streptomycin. The rat VSMCs (A10 cell line) were purchased from the American Type Culture Collection (ATCC). A10 cells were cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% FBS and 100 U/mL penicillin and 100 U/mL streptomycin. All cells were cultured under 37 °C and 5%
2.9. Flow cytometric analysis

Macrophages were seeded into the lower section of 24 mm Transwell chambers at a density of 2 × 10^5/well in 1640 medium with 15% heat-inactivated FBS. The membranous scaffolds (PCL, PCL-epECM/H, PCL-epECM/H-IL-4) were placed in upper chambers with 8 μm pores (Costar, Cambridge, MA) (n = 3). After 24 h, macrophages were harvested by cell scraping and washed with PBS, then incubated with FITC-conjugated anti-CD68 (BioLegend, USA) antibody, and APC-conjugated anti-CD206 (BioLegend, USA) antibody for 45 min. Flow cytometry was performed on a FACS Calibur (BD, USA).

2.10. Cytokine measurement

Macrophages were seeded on different membranous scaffolds (PCL, PCL-epECM/H, PCL-epECM/H-IL-4) at a density of 5 × 10^5 cells/well with 1640 medium supplemented with 5% heat-inactivated FBS in 6-well plates. The macrophages seeded on blank plates were set as negative control (labeled as TCP). The macrophages seeded on blank plates with the addition of 10 ng/mL IL-4 into the medium were set as the positive control (labeled as IL-4) (n = 3). The supernatants were collected at 1 day and 3 days, centrifuged at 250 g for 8 min and immediately stored at −80 °C. Then the magnetic beads (Luminex) multiplexed cytokine assays was performed, according to the manufacturer’s recommendations. Magnetic beads with 7 distinct spectral signatures (regions) were coupled to cytokine-specific capture antibodies without cross-reactivity. Data were collected on Luminex 200 analyzer and analyzed using Bioplex Manager software (Bio-Rad). 4P regression algorithms was used to determine cytokine concentrations, values below the lower limit of quantification were set to zero.

2.11. In vitro macrophage-polarisation assay

Macrophages were seeded on different membranous scaffolds (PCL, PCL-epECM/H, PCL-epECM/H-IL-4, n = 5) in 48-well plates at a density of 5 × 10^5 cells/well. After 1 and 3 days of culturing, the macrophages were fixed with 4% PFA (Solarbio, China) and blocked using 5% normal goat serum (Zhongshan Golden Bridge Bio-Tech, China) for 45 min at room temperature, and then incubated with primary antibodies for mouse anti-CD68 (1:100, Abcam, ab31630, USA), and rabbit anti-Mannose Receptor antibodies (1:200, Abcam, ab64693, USA) overnight at 4 °C. Alexa Fluor 488 goat anti-mouse IgG (1:200, Invitrogen, USA) and Alexa Fluor 546 goat anti-rabbit IgG (1:200, Invitrogen, USA) were applied to react with the primary antibodies for 2 h at room temperature. Finally, the nuclei were stained with DAPI. The DAF-FM in microplates was detected at 4 °C. Alexa Fluor 488 goat anti-mouse IgG (1:200, Invitrogen, USA) were applied to react with the primary antibodies for mouse anti-α-SMA (1:100, Abcam, ab7817, USA) and rabbit anti-calponin 1 antibodies (1:200, Abcam, ab46794, USA) overnight at 4 °C. Alexa Fluor 488 goat anti-mouse IgG (1:200, Invitrogen, USA) and Alexa Fluor 546 goat anti-rabbit IgG (1:200, Invitrogen, USA) were applied to react with the primary antibodies for 2

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2.13. Macrophage-VSMCs interaction experiments

We used a transwell system to investigate the interaction of macrophage-VSMCs. For the migration assay, the upper chambers were seeded with VSMCs at a density of 1 × 10^5 cells/well and the lower chambers were placed with different scaffolds (PCL, PCL-epECM/H, or PCL-epECM/H-IL-4) and seeded with macrophages at a density of 5 × 10^5 cells/well. The supernatant was taken out from each well and added to a 96-well plate. The optical density (OD) value was measured at 450 nm using a microplate reader (Bio-Rad, USA).
h at room temperature. Finally, the nuclei were stained with DAPI (Southern Biotech, England) and observed with a LSCM (Leica TCS SP8, Germany). For the proliferation assay, the upper chambers were placed with different scaffolds (PCL, PCL-epECM/H, or PCL-epECM/H-IL-4) seeded with macrophages at a density of $5 \times 10^4$ cells/well and the lower chambers were seeded with VSMCs at a density of $8 \times 10^4$ cells/well ($n = 3$). At the predetermined time points, CCK-8 dilution in DMEM (1:10) was added to each well after removing the media. Upon incubation for 1 h at 37°C, 100 µL supernatant was taken out from each well and added to a 96-well plate. The optical density (OD) value was measured at 450 nm using a microplate reader (Bio-Rad, USA).

2.14. Animal surgery and grouping scheme

69 adult male Sprague-Dawley rats (aged 8–10 weeks with a weight range of 280–320 g) were used to evaluate vascular regeneration in vivo (Table 1). The animals were anesthetized deeply by intraperitoneal injection of ketamine (40 mg/kg)-xylazine (5 mg/kg)-acepromazine (1 mg/kg), and isoflurane gas was used as assisted anesthesia throughout the procedure. Heparin was injected through the tail vein before surgery. The abdominal aorta was isolated, clamped, and transected. The grafts (2.0 mm in inner diameter, 400–450 mm length) were implanted in an end-to-end way with 8–10 interrupted stitches using 9–0 monofilament nylon sutures (Lingqiao, Ningbo, China). The skin was closed with 3–0 monofilament nylon sutures (Lingqiao, Ningbo, China). All the needle point types were round bodied and basic shapes were 3/8 Circle. No anti-coagulation drug was administered to the rats after surgery. All rats were housed and fed routinely and euthanized by heart perfusion of physiological saline after being deeply anesthetized at different time points (1 wk, 2 wks, 4 wks and 12 wks). The rats were examined by Doppler ultrasound (Vevo 2100 System, Visualsonics, Canada) before euthanizing.

2.15. Histological analysis

The explanted grafts were cut in two parts at the half position of the long axis. One part of the grafts was put into OCT compound and snap-frozen by liquid nitrogen for longitudinal sections for immunofluorescence staining, and the other part was longitudinally cut into two pieces. After observation under a stereomicroscope (LEICA S8AP0, Germany), one-piece was embedded in OCT snap-frozen by liquid nitrogen for frozen cross-section. The sections were stained with hematoxylin and eosin (H&E), Masson’s trichrome, Verhoeff’s, safranin O and then observed under upright microscope (Leica DM3000, Germany). The other part was longitudinally cut into two pieces. After observation under a stereomicroscope (LEICA S8AP0, Germany), one-piece was embedded in OCT snap-frozen by liquid nitrogen for longitudinal sections for immunofluorescence staining, and the other piece was fixed by 2.5% glutaraldehyde for the SEM (Hitachi, s-4800, Japan) examination.

2.16. Immunofluorescence staining

Frozen sections were fixed in acetone at −20°C for 10 min, air-dried, and rinsed with 0.01 mM PBS three times. Triton X–PBS (0.1%) was used for intracellular antigen staining to permeate the cell membrane. Slides were blocked with 5% normal goat serum for 45 min at 4°C, and then were incubated with the following antibodies for 12 h at 4°C. The mouse anti-Cd68 (1:200, Abcam, ab31630, USA), rabbit anti-cD206 (ab64693), anti-TNF-α (ab183218) and rabbit anti-iNOS (ab15323) antibodies were used to mark the macrophages. The mouse anti-α-SMA (1:100, Abcam, ab78177, USA), mouse anti-MYH11 (1:100, Santa Cruz, sc-6956, USA) rabbit anti-Ki67 (1:100, Abcam, ab15580, USA) and rabbit anti-calponin 1 (1:200, Abcam, ab46794, USA) antibodies were used to mark the VSMCs. The rabbit anti-collagen I (1:200, Abcam, ab266043, USA), rabbit anti-collagen III (1:200, Abcam, ab7778, USA) and rabbit anti-elastin (1:200, Abcam, ab21610, USA) antibodies were used to mark the extracellular matrix deposition. The rabbit anti-Von Willebrand Factor (1:200, Abcam, ab6994, USA) antibody was used to mark the ECs. After completing the incubation with primary antibodies, the slides were washed 8 times with PBS and then incubated with appropriate secondary antibodies for 2 h at room temperature. The cell nuclei were stained with DAPI (Southern Biotech, England). The slides were then observed under a fluorescence microscope (Zeiss Axio Imager Z1, Germany).

2.17. Statistical analysis

All quantitative results were obtained from at least three independent experiments. Data were expressed as the mean ± standard deviation (SD). GraphPad Prism Software Version 8.0 (San Diego, CA, USA) was used for statistical analysis. Single comparisons were carried out using an unpaired student’s t-test. Multiple comparisons were carried out using one-way analysis of variance (ANOVA) and Tukey’s post-hoc test. Multivariant comparisons were carried out using two-way ANOVA with Tukey’s post-hoc test. The minimum significance levels were set at *p < 0.05, **p < 0.001, and ***p < 0.0001; ns: no significance.

3. Results

3.1. Fabrication and characterization of bioactive hybrid scaffolds

Firstly, pECM was obtained from the donated human placenta via decellularization treatments. Stereoscopic images showed that the placental tissue changed from red to white and translucent after decellularization. Scanning electron microscopy (SEM) examination showed little change in the microscopic structure after decellularization. DAPI staining demonstrated that almost all of the nuclei were removed (Fig. 1A). DNA residue in pECM (42.3 ± 2.6 ng/mg) was lower than the minimal criteria for acellular products (50 ng/mg, Fig. S1A) [28]. Hematoxylin and eosin (H&E) staining showed that the pECM preserved the abundant components of ECM (Fig. S1B). Next, pECM nanofibers were fabricated by electrospinning (pECM). SDS-PAGE illustrated that most protein bands were retained in pECM after electrospinning (Fig. 1B). Further proteomics analysis revealed that proteins involved in angiogenesis, immune regulation and tissue remodeling were still reserved in pECM (Fig. 1C). In addition, rich types of collagen, including fibrillar, fibril-associated, sheet-forming, etc. in pECM and pECM could be detected (Fig. S1C). Quantitative experiments also showed no significant difference in collagen content between pECM and pECM, which were higher than those in native tissue (Fig. S1D). Both pECM and pECM scaffolds contain plentiful laminin and fibronectin (Fig. S1E). Safranin O staining showed that a large number of GAGs remained in pECM (Fig. S1F), and GAGs content in pECM was higher than that of pECM, close to that of natural tissue (Fig. S1G).

The PCL microfiber and pECM nanofiber hybrid membranous or tubular scaffolds were fabricated by co-electrospinning (Fig. 1D). There was no significant difference in the diameter of pECM nanofibers produced by using different flow rates (Figs. S2A and C). The hydrophilicity of the hybrid scaffolds and the density of the pECM nanofibers were enhanced as the flow rate increase (Figs. S2B and C). Fourier transform infrared spectroscopy (FTIR) spectra evaluated changes in protein composition. Both pECM and pECM exhibited prominent characteristic peaks of peptide group (3000 cm−1), amide I band and amide II band at 1630 cm−1 and 1535 cm−1 (Fig. 1E), with no noticeable
Fig. 1. Fabrication and characterization of bioactive hybrid scaffolds. A) Representative stereoscopic images, SEM and DAPI staining images of human placenta tissue before (Placenta) and after (pECM) decellularization. B) SDS-PAGE showed total protein extracted from pECM and epECM. C) Heatmap of proteins involved in angiogenesis, immunoregulation, and tissue remodeling in pECM and epECM. D) Schematic illustration of the fabrication process of hybrid vascular grafts by co-electrospinning. E) FTIR spectra of the pECM, epECM, PCL, and PCL-epECM scaffolds. F) Confocal images and SEM images of PCL and PCL-epECM membranous and tubular scaffolds. G) TGA curve of PCL and PCL-epECM scaffolds. H) In vitro degradation of PCL scaffolds and PCL-epECM scaffolds. I) Schematic illustration of the active modification process of hybrid scaffolds. J) Representative stress-strain curves of different grafts. K-M) The maximum stress and strain at break and elastic modulus of different grafts. N) Toluidine blue staining images and heparin loading statistics of PCL and PCL-epECM/H scaffolds. O) In vitro release kinetics of IL-4 on the PCL-epECM/H-IL-4 scaffolds. Scale bars: A) first column, 5 mm; second column and third column, 50 μm column. F) first column, 50 μm; second column and fourth column, 30 μm; third column, 300 μm.

Fig. 2. Hemocompatibility evaluation of PCL, PCL-epECM/H, and PCL-epECM/H-IL-4 vascular grafts. A) Schematic representation of the AV shunt process and brightfield photographs of different vascular grafts after AV shunt. B) Stereomicroscopy images of the luminal surfaces of different grafts. C) SEM images of the luminal surfaces of different grafts. D) Mepacrine staining showed the platelet adhesion on the lumen of different grafts. E) The number of adherent platelets. F-I) Quantification of FIB, APTT, TT, PT of different grafts. Scale bars: B) 2 mm, C) 10 μm, D) 50 μm.
difference. In PCL-epECM, both characteristic peaks of protein and PCL (C=O, C-C, 1720 cm⁻¹, 1140 cm⁻¹) were detected, further verified the negligible destructive effect of electrospinning and the successful composite of two materials. Both confocal microscopy and SEM images showed that PCL microfiber and epECM nanofiber cross-distributed in the hybrid membranous and tubular scaffolds with the obviously diverse diameter (Fig. 1F). The diameter of PCL microfiber (red-stained in fluorescent image) in pure PCL scaffold (7.8 ± 0.9 μm) and hybrid scaffold (7.4 ± 1.3 μm) presented no noticeable difference. After electrospinning, the epECM nanofibers (green-stained in fluorescent image) remained nanoscale with a diameter of 0.7 ± 0.4 μm. Thermal gravimetric analysis (TGA) showed that the onset of decomposition temperature (T onset) was similar for both scaffolds. However, the PCL-epECM group still had about 15% of the remaining mass at 800 °C, which further proved the successful fabrication of the hybrid scaffolds (Fig. 1G). Furthermore, the degradation rate of PCL-epECM scaffolds was faster than PCL scaffolds under the condition of 5 M NaOH treatment. The PCL scaffolds still retained about 40% of their initial mass, while PCL-epECM scaffolds were completely degraded at 9 h (Fig. 1H). The incorporation of epECM nanofibers significantly reduced the scaffolds’ water contact angle (WCA). The pore size among the microfibers in pure PCL scaffolds and PCL-epECM scaffolds was 14.3 ± 6.1 μm and 13.7 ± 5.0 μm, with no significant differences. While the pore size among the microfibers and nanofibers in PCL-epECM scaffolds was 7.9 ± 3.3 μm (Fig. S2D). The stress-strain curves of PCL grafts, PCL-epECM/ H grafts, and PCL-epECM/H-IL-4 grafts maintained a similar trend (Fig. 1J). The maximum stress and strain at the break of the PCL grafts were higher than that of the PCL-epECM/h grafts and PCL-epECM/H-IL-4 grafts (Fig. 1K and L), and the PCL grafts’ elastic modulus was lower than that of PCL-epECM/h grafts and PCL-epECM/H-IL-4 grafts (Fig. 1M), displaying no significant differences among three groups. Live/dead staining of RAECs confirmed that hybrid scaffolds (PCL-epECM/H and PCL-epECM/H-IL-4) possessed higher biocompatibility than PCL scaffolds (Figs. S3A and B). CCK-8 assay demonstrated that hybrid scaffolds (PCL-epECM/H and PCL-epECM/H-IL-4) can effectively promote the proliferation of RAECs and VSMCs, which further verified that hybrid scaffolds had stronger bioactivity than PCL scaffolds (Figs. S3C and D).

Heparin was cross-linked to the epECM surface via EDC/NHS, and then IL-1 was loaded through electrostatic interactions, as shown in Fig. 11. Toluindine blue staining showed that the PCL-epECM/H scaffolds were colored in blue-violet, while the pure PCL scaffolds were still milky-white. The surface heparin density on the PCL-epECM/H was about 18 μg/cm² (Fig. 1N). Further, the IL-4 on PCL-epECM/H-IL-4 scaffolds could achieve sustained release for 14 days with a cumulative release of 20.8% ± 1.5% (Fig. 1O).

3.2. Hemocompatibility evaluation of different vascular grafts

The grafts’ hemocompatibility was evaluated by the rat arteriovenous shunt assay (Fig. 2A). Stereomicroscopy images showed that the wall and lumen of the PCL grafts were entirely red, while the PCL-epECM/H grafts and PCL-epECM/H-IL-4 grafts were partially red (Fig. 2A and B). Further, as observed by SEM images, plenty of platelets, fibrin and blood cells were distributed on the lumen of PCL grafts, but little on heparin modified grafts (Fig. 2C). Mipaline staining showed that the modification of heparin significantly inhibited the platelets adhesion (Fig. 2D), which was consistent with the results of SEM. The number of adhered platelets on heparin modified grafts was considerably lower than PCL grafts (Fig. 2E). Additionally, the blood fibrinogen (FIB), activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT) were detected after co-incubation of the grafts with normal human blood. FIB adhesion in heparin-modified grafts was vastly less than in PCL grafts (Fig. 2F). The APTT, TT, PT in heparin-modified grafts were significantly longer than PCL grafts, no significant differences were found between PCL-epECM/H grafts and PCL-epECM/H-IL-4 grafts (Fig. 2G–I).

3.3. The effects of different scaffolds on the macrophages polarization

We investigated phenotype, morphology, and cytokine secretion of macrophages on various scaffolds. Flow cytometry gating strategies for macrophages and CD206+ cells were quantitatively analyzed (Figs. S4A and B). Flow cytometry analysis showed that the number of CD206+ cells in the PCL-epECM/H scaffolds increased by over 7% compared with the PCL scaffolds. The number of CD206+ cells in PCL-epECM/H-IL-4 scaffolds increased by nearly 11% compared with the PCL-epECM/H scaffolds, and over 7% higher than the IL-4 positive control group (Fig. 3A). Immunofluorescence co-staining (CD68, CD206) indicated that the number of CD206+ cells in PCL-epECM/H and PCL-epECM/H-IL-4 scaffolds was significantly higher than that in PCL scaffolds at 1 day and 3 days. The number of CD206+ cells in PCL-epECM/H-IL-4 scaffolds was higher than PCL-epECM/H scaffolds, although no noticeable difference was observed at 3 days (Fig. 3B). The ratio of CD206/CD68 also presented a similar trend (Fig. 3C). SEM images showed that the macrophages in PCL scaffolds were mostly spherical without spreading, while the cells in the other two scaffolds showed spreading and elongated morphology. The macrophages in the PCL-epECM/H-IL-4 scaffolds had the highest elongation factor (i.e., aspect ratio), and the trend of cell elongation increased with the extension of time (Fig. 3D and E). Furthermore, inflammatory cytokine measurements were performed on supernatants to evaluate soluble cytokines secreted by macrophages on different scaffolds. We used an in-house multiplexed magnetic bead-based assay (Luminex) to measure the following 7 cytokines: IL-10, IL-4, IL-5, IL-2, IL-1β, IFN-γ, TNF-α. The concentration of anti-inflammatory factor IL-10 from PCL-epECM/H-IL-4 scaffolds and PCL-epECM/H scaffolds were significantly higher than PCL scaffolds at 1 day. The expression level of anti-inflammatory factor IL-4 from PCL-epECM/H-IL-4 scaffolds was markedly higher than PCL scaffolds and PCL-epECM/H scaffolds at 1 day. The secretion of pro-inflammatory factor IL-5 from PCL-epECM/H-IL-4 scaffolds was lower than PCL scaffolds and PCL-epECM/H scaffolds at 1 day, although no significant differences were found among the three groups. The concentration of pro-inflammatory factor IL-2 from PCL-epECM/H-IL-4 scaffolds was significantly lower than other groups. The concentration of pro-inflammatory factor IL-1β from PCL-epECM/H-IL-4 scaffolds was considerably lower than PCL scaffolds, although no significant differences were found among PCL-epECM/H-IL-4 scaffolds and other groups. The expression level of typical pro-inflammatory factors (IFN-γ and TNF-α) PCL-epECM/H-IL-4 scaffolds was significantly lower than PCL scaffolds and PCL-epECM/H scaffolds (Fig. 3F). The expression level of IL-10 among all groups showed no apparent difference at 3 days. The expression level of IL-4 from PCL-epECM/H-IL-4 scaffolds was still higher than PCL scaffolds and PCL-epECM/H scaffolds at 3 days, although without significant difference. The concentration of pro-inflammatory factors (including IL-5, IL-2, and TNF-α) among all groups showed no significant difference. The expression level of IL-1β and IFN-γ from PCL-epECM/H-IL-4 scaffolds was lower than in other groups at 3 days (Fig. 3G).

3.4. Effect of polarized macrophages on migration, NO generation and tube formation of rat aortic endothelial cells (RAECs) in vitro

We used a transwell system to investigate macrophage-RAECs interaction, in which the upper chambers were placed with different scaffolds and seeded with macrophages and the lower chambers were seeded with RAECs (Fig. 4A). Bright field pictures and corresponding statistical analysis showed that the healing speed of RAECs in the PCL-epECM/H-IL-4 group was significantly higher than that in other groups at 10 h and 20 h (Fig. 4B and C). Subsequently, we also explored the effect of macrophages cultured on different scaffolds on NO production from RAECs (Fig. 4D). DAF-FM fluorescence images and fluorescence intensity quantification confirmed that NO production from RAECs in the PCL-epECM/H-IL-4 group and PCL-epECM/H group was higher than the PCL group (PCL-epECM/H-IL-4: PCL = 1.8 folds, PCL-
Fig. 3. The polarization of macrophages on PCL, PCL-epECM, and PCL-epECM/H-IL-4 scaffolds. A) Flow cytometry analysis of CD206+ macrophages on different scaffolds at 1 day. B) Double immunofluorescence staining images of nonpolarized macrophages (CD68, green) and anti-inflammatory macrophages (CD206, red) cultured on different scaffolds at 1 day and 3 days. C) The ratio of CD206 to CD68 positive cells cultured on different scaffolds at 1 day and 3 days. D) SEM showing the morphology of macrophages cultured on different scaffolds at 1 day and 3 days. E) The elongation factor of macrophages in different scaffolds at 1 day and 3 days. F, G) The concentration of inflammatory cytokines in the supernatant of macrophages cultured on different scaffolds for 1 day and 3 days. Scale bars: B) 50 μm, D) 5 μm.
Fig. 4. Effect of polarized macrophages on migration, NO generation and tube formation of RAECs in vitro. A) Schematic illustration of co-culture experiments of RAECs migration mediated by macrophages cultured on different scaffolds. B, C) Representative images and corresponding quantitative analysis of scratch-wound migration assay for RAECs mediated by macrophages cultured on different scaffolds at 0 h, 10 h, and 20 h. D) Schematic illustration of co-culture experiments of NO production of RAECs mediated by macrophages cultured on different scaffolds. E, F) Representative immunofluorescence images and corresponding relative quantification of NO production in RAECs mediated by macrophages cultured on different scaffolds detected by using the DAF-FM probe (green) at 3 days. Cell nuclei stained by DAPI (blue). G) Schematic illustration of RAECs tube formation stimulated by polarized macrophages on different scaffolds. H) F-actin immunostaining pictures and brightfield images of tube formation of RAECs stimulated with conditioned medium collected from macrophages cultured on different scaffolds after 4 h. I-L) Quantitative statistics of the number of nodes, meshes, junctions and branching length of RAECs after 4 h incubation with macrophages conditioned medium.

Scale bars: B) 100 μm, E) 20 μm, H) 100 μm.
epECM/H: PCL = 1.7 folds), although there was no significant difference between the PCL-epECM/H-IL-4 and PCL-epECM/H group (Fig. 4E and F). In addition, we further explored the effect of the conditioned medium obtained from macrophages seeded on different scaffolds on RAECs tube formation (Fig. 4G). The tube formation in the PCL-epECM/H-IL-4 group was increased compared with PCL and PCL-epECM/H groups (Fig. 4H). The statistical results showed that the node number, ring number, branch point number, and branch length of the tube structure in the PCL-epECM/H-IL-4 group were higher than other groups, though no obvious difference was found between the PCL-epECM/H-IL-4 and PCL-epECM/H groups (Fig. 4I-L). CCK-8 assay revealed that RAECs in different scaffolds could maintain proliferation activity, although there was no significant difference among the three scaffolds (Fig. 5S). The PCL-epECM/H-IL-4 group improved RAECs migration, NO generation, and tube formation.

3.5. Effects of polarized macrophages on migration and maturation of vascular smooth muscle cells (VSMCs) in vitro

A transwell system was used to investigate macrophage-VSMCs interaction, in which the upper chambers were seeded with VSMCs and the lower chambers were placed with different scaffolds seeded with macrophages (Fig. 5A). Crystal violet staining and further statistical results showed that the number of migrated VSMCs in PCL-epECM/H-IL-4 groups and PCL-epECM/H groups was significantly higher than that in PCL groups (PCL-epECM/H-IL-4: PCL = 3.5 folds, PCL-epECM/H: PCL = 3.6 folds), although there was no significant difference between the two groups (Fig. 5B and C). Afterward, we also used a transwell system to explore the effect of macrophages cultured on different scaffolds on VSMCs maturation (Fig. 5D). Immunofluorescence staining and corresponding fluorescence quantitative statistics displayed the macrophages cultured on different scaffolds had little effect on the expression of α-SMA protein in VSMCs (Fig. 5E and F), but the PCL-epECM/H-IL-4 group could effectively up-regulate the expression of contractile protein (Calponin) (Fig. 5E, G). Furthermore, we found that the VSMCs can proliferate over time in all groups (Fig. 56).

3.6. Macrophage behavior in different vascular grafts remodeling process

We further evaluated the infiltration and polarization of macrophages in different vascular grafts at various time points (1 wk, 2 wks, 4 wks, 12 wks) in vivo. The number of pan-macrophages (M0), pro-inflammatory and anti-inflammatory macrophages were identified by immunofluorescent staining for CD68, TNF-α, iNOS and CD206, respectively. CD68+ macrophages were mainly distributed in the graft wall all the time. The number of CD68+ cells showed a decreasing trend, and there was no significant difference among all grafts at 1 wk and 12 wks. The number of CD68+ cells in PCL-epECM/H-IL-4 grafts was lower than that of the other two grafts at 2 wks and 4 wks, although no significant difference was detected between the PCL-epECM/H grafts and PCL-epECM/H-IL-4 grafts at 2 wks (Fig. 6A, E). The number of TNF-α+ cells increased first and then decreased in PCL grafts, and the other two grafts showed a gradually decreasing trend. At different time points, the number of TNF-α+ cells in PCL-epECM/H-IL-4 grafts was significantly lower than in the PCL grafts, although no significant difference was observed between the PCL-epECM/H-IL-4 and PCL-epECM/H grafts (Fig. 6B, F). The number of iNOS+ cells decreased over time. The number of iNOS+ cells in the PCL-epECM/H-IL-4 grafts was significantly lower than that in the PCL grafts at 1 wk, 2 wks and 4 wks, although no significant difference was detected between the PCL-epECM/H-IL-4 and PCL-epECM/H grafts, and no significant difference was found among three grafts at 12 wks (Fig. 6C, G). The number of CD206+ cells showed a steady downward trend over time. The number of CD206+ cells in the PCL-epECM/H-IL-4 grafts was significantly higher than that in the other two grafts at 1 wk and 2 wks. The apparent difference only existed between PCL-epECM/H-IL-4 grafts and PCL grafts at 4 wks, and there was no significant difference among all grafts at 12 wks (Fig. 6I). Co-staining also demonstrated the same result (Fig. 57).

3.7. Patency and endothelialisation of different vascular grafts

Patency of implanted vascular grafts is a prerequisite for tissue regeneration. No apparent thrombus formation, stenosis, aneurysms, or bleeding could be detected by the Doppler ultrasound test in all groups from 1 wk to 12 wks. However, 20% of the PCL grafts had a small amount of coagulation on the luminal surface at 1 wk and 2 wks post-implantation, while the other grafts’ luminal surfaces were clean and smooth (Fig. 58A). The blood flow velocity of PCL-epECM/H-IL-4 grafts was close to that of natural rat abdominal aorta within 12 wks (782 ± 33 mm/s) and higher than that of PCL grafts (Figs. 58B-E).

Rapid endothelialization can inhibit thrombosis and enhance long-term patency. No bare fibers can be observed in the lumen of PCL-epECM/H-IL-4 grafts by SEM after 1 wk of implantation, while numerous bare fibers distributed in the lumen of the other two grafts (Fig. 59A). After 2 wks of implantation, the lumen of the PCL-epECM/H grafts and the PCL-epECM/H-IL-4 grafts exhibited a smooth and flat morphology, and there was still a certain area of bare fibers in the PCL grafts (Fig. 59B). Although the PCL grafts were partially covered by neotissue at the anastomosis after 4 wks of implantation, a large area of fibers could be observed in the quarter and mid positions. Although the PCL-epECM/H grafts was covered with neointima, the typical endothelial cells with cobblestone-like arrangement could be observed in the mid position. However, the lumen of PCL-epECM/H-IL-4 grafts was covered by a layer of cobblestone-like endothelial cells parallel to the direction of blood flow even at the mid position (Fig. 57A). The vWF immunostaining was used to evaluate endothelial coverage and capillaries formation in the grafts wall. Consistent with the SEM observation, a monolayer of vWF+ cells in the PCL-epECM/H-IL-4 grafts covered the lumen (89.6 ± 1.9%), whose coverage rate was higher than the other two grafts at 4 wks (PCL-epECM/H: 82.9 ± 4.5%, PCL: 67.4 ± 3.3%), although no significant difference was found between the PCL-epECM/H grafts and PCL-epECM/H-IL-4 grafts (Fig. 7B, E). At 12 wks, the PCL grafts were also covered by a neointima, but the cell morphology differed from the native endothelial cells, especially in the mid position. In the PCL-epECM/H-IL-4 grafts and the PCL-epECM/H grafts, the cobblestone-shaped endothelial cells were arranged along the blood flow direction in the entire lumen (Fig. 7C). vWF immunofluorescence staining and statistical data showed that the vWF+ cell coverage rate of PCL-epECM/H-IL-4 grafts (98.9 ± 1.7%) and PCL-epECM/H grafts (93.6 ± 2.8%) was higher than that of PCL (90.3 ± 2.2%) at 12 wks (Fig. 7D and E). Immunofluorescence staining and statistical data also showed that CD31+ cell covered the lumen of the grafts, and the coverage rate of PCL-epECM/H-IL-4 grafts was the highest among the three grafts at 4 and 12 wks (Figs. 59C-E). In addition, the number of capillaries in the wall of PCL-epECM/H and PCL-epECM/H-IL-4 grafts were higher than that of PCL grafts at 4 wks and 12 wks, although there was no significant difference between the PCL-epECM/H and PCL-epECM/H-IL-4 grafts (Fig. 7F).

3.8. VSMCs regeneration within different vascular grafts in vivo

Contractile VSMCs play an essential role in the mechanical properties, vasoconstriction, and relaxation of the artery. Immunofluorescence staining showed that α-SMA+ cells were mainly distributed in the graft wall at 1 wk and 2 wks, and layers of spindle-shaped α-SMA+ cells principally dispersed in a circumferentially oriented direction in the lumen at 4 wks and 12 wks (Fig. 59A). The number of α-SMA+ cells in...
Fig. 5. Effects of polarized macrophages on migration and maturation of VSMCs in vitro. A) Schematic illustration of co-culture experiments of VSMCs migration mediated by macrophages cultured on different scaffolds. B, C) Representative images and corresponding quantitative analysis of migrated VSMCs affected by macrophages on different scaffolds at 24 h. D) Schematic illustration of co-culture experiments of VSMCs maturation mediated by macrophages cultured on different scaffolds. E) Double immunofluorescence staining images of α-SMA protein (green) and Calponin protein (red) in VSMCs cultured on different scaffolds after 7 days. Cell nuclei stained by DAPI (blue). F, G) The fluorescence intensity of α-SMA and Calponin in VSMCs on different scaffolds. Scale bars: B) 100 μm, E) 50 μm.
Fig. 6. Macrophages infiltration and polarization within different grafts in vivo. A-D) Representative immunofluorescent images of the nonpolarized macrophages (CD68, green), pro-inflammatory macrophages (TNF-α, red; iNOS, red), anti-inflammatory macrophages (CD206, red). Cell nuclei stained by DAPI (blue). E-H) Quantification of the number of CD68⁺ cells, TNF-α⁺ cells, iNOS⁺ cells and CD206⁺ cells. I) The anti-inflammatory/pro-inflammatory macrophages ratio at 1 wk, 2 wks, 4 wks and 12 wks post-implantation. Scale bar: 50 μm.
PCL-epECM/H and PCL-epECM/H-IL-4 grafts was higher than in PCL grafts from 1 wk to 12 wks, and noticeable differences exist at 4 wks and 12 wks (Fig. 8 E). The thickness of the smooth muscle layer in both PCL-epECM/H grafts and PCL-epECM/H-IL-4 grafts were significantly higher than that in the PCL grafts at 4 wks (PCL-epECM/H-IL-4: PCL = 3.1 folds, PCL-epECM/H: PCL = 2.8 folds) and 12 wks (PCL-epECM/H-IL-4: PCL = 1.8 folds, PCL-epECM/H: PCL = 1.8 folds), although there was no significant difference between the PCL-epECM/H and PCL-epECM/H-IL-4 grafts (Fig. 8F). Calponin+ cells were nearly absent in the graft wall at 1 wk, sporadically distributed in the graft wall at 2 wks, and abundantly dispersed in the lumen at 4 wks and 12 wks (Fig. 8B). The number of Calponin+ cells of PCL-epECM/H-IL-4 grafts was higher than the other two grafts at 2 wks, 4 wks and 12 wks (Fig. 8G). Similarly, MYH+ cells were sporadically distributed in the graft wall at 1 wk and 2 wks, and abundantly dispersed in the lumen at the 4 wks and 12 wks (Fig. 8C). There was no significant difference in the number of MYH+ cells among three grafts at 1 wk and 2 wks, and the number of MYH+ cells of PCL-epECM/H-IL-4 and PCL-epECM/H were significantly higher than PCL grafts at 4 wks and 12 wks (Fig. 8H). Co-immunostaining of Ki67+ and α-SMA+ was used to evaluate smooth muscle layer stability. The number of double-positive cells in the PCL-epECM/H-IL-4 grafts was significantly higher than that in the other two grafts at 1 wk, and a small amount of double-positive cells were distributed in all grafts with no significant difference at 2 wks, 4 wks and 12 wks (Fig. 8D, I).

3.9. ECM deposition in different vascular grafts

New ECM deposition including collagen, elastin, and GAGs of explanted vascular grafts was examined. H&E staining showed that the ECM was primarily distributed in the graft wall, and PCL-epECM/H grafts and PCL-epECM/H-IL-4 grafts exhibited increased cellular infiltration and ECM deposition than PCL grafts at 1 wk and 2 wks. The ECM
Fig. 8. VSMCs regeneration within different vascular grafts in vivo from 1 wk to 12 wks post-implantation. A-D) Representative immunofluorescent images of the α-SMA (green), Calponin (red), MYH (green), α-SMA (green) and Ki67 (red) of grafts’ cross-sections. Cell nuclei stained by DAPI (blue). E, G, H) The fluorescence intensity of α-SMA, Calponin and MYH in different grafts. F) The thickness of smooth muscle layer in different grafts at 4 and 12 wks. I) The number of Ki67 and α-SMA double positive cells in different grafts. Scale bar: 100 μm.
in the PCL grafts was only distributed in the graft wall, while it was
distributed in both wall and lumen of PCL-epECM/H grafts and PCL-
epECM/H-IL-4 grafts at 4 and 12 wks. ECM deposited within the
lumen of both grafts exhibited a circumferentially oriented arrange-
ment. Additionally, ECM deposition showed an increasing trend from 1
wk to 12 wks. Masson and Sirius Red staining confirmed that the
collagen in the PCL-epECM/H-IL-4 grafts was denser and more orderly
than the other two grafts, and the collagen deposition also increased
over time. Immunofluorescence staining further confirmed the distri-
bution of collagen I and collagen III. Both Verhoeff staining and elastin
immunostaining disclosed continuous wave-like elastin in the PCL-
epECM/H-IL-4 grafts. Safranin O staining indicated abundant GAGs
deposition in the PCL-epECM/H-IL-4 grafts and PCL-epECM/H grafts
(Fig. 9A & Fig. S10A & Fig. S11). Semi-quantitative results showed that
collagen I, collagen III, elastin, and GAGs in PCL-epECM/H-IL-4 grafts
were significantly higher than those in PCL grafts at 4 wks and 12 wks,
although no significant difference was detected between PCL-epECM/H
grafts and PCL-epECM/H-IL-4 grafts (Fig. 9B-E & Fig. S10B-E).

4. Discussion

To meet the requirement of both anticoagulated bioactivity and

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**Fig. 9.** ECM deposition and organization within different vascular grafts at 12 wks post-implantation. A) Microscopic images of cross-sections stained with H&E for ECM, Masson’s trichrome and Sirius red for collagen, immunofluorescence staining for collagen I (red) and collagen III (red), Verhoeff’s and immunofluorescence staining for elastin, safranin O for GAGs. B-E) Relative quantification of Collagen I, Collagen III, Elastin, and GAGs expression. Scale bar: 100 μm.
adequate mechanical strength of vascular scaffolds, we fabricated PCL microfiber and pECM nanofiber hybrid scaffolds, and heparinized pECM and further functionalized with IL-4. Compared with PCL scaffolds, the PCL-epECM/H-IL-4 scaffolds exhibited anti-coagulant capabilities, promoted macrophages’ polarization to anti-inflammatory phenotype, and further enhanced the migration and maturation of RAECs and VSMCs in vitro. The PCL-epECM/H-IL-4 scaffolds enhanced constructive remodeling as evidenced by high anti-inflammatory/pro-inflammatory macrophage ratio, rapid endothelialization, high expression levels of smooth muscle contractile proteins, and improved ECM deposition in vivo. This study provided a new strategy for the construction of SDVGs and other scaffolds used for tissue repair.

Synthetic biodegradable polymer materials have favorable mechanical strength, controllable degradation and processing properties, which can meet the mechanical and preparation requirements of SDVGs. Our laboratory and other groups developed the PCL microfiber SDVGs, which exhibited improved tissue regeneration [4,27,29]. However, the biological inertness and lack of reactive sites of synthetic biodegradable materials showed incompetent vascular constructive regeneration. The dECM materials possessed enhanced biocompatibility and bioactivity, as well as abundant functional modification reaction sites [30]. Some decellularized perinatal tissues (e.g., amniotic membrane and umbilical cord) had been widely used clinically for over a century [31,32]. As the main component of perinatal tissues, the placenta played a vital role in exchanging nutrients and metabolites, immune regulation, cytokine secretion and hematopoiesis [33]. The pECM composed of various protein and bioactive factors promoted angiogenesis, neurogenesis, anti-inflammatory, anti-microbial and immune privilege [34]. The mass spectrometric analysis identified 3395 proteins in pECM, which was twice as much as commonly used Matrigel (1617 proteins), and far more than the porcine bladder (129 proteins) [35–37]. Our results also showed that pECM was a positive factor for remodeling. Compare to PCL scaffolds, RAECs migration, NO production, tube formation, SMCs migration, and the Calponin expression were all enhanced in hybrid scaffolds (Figs. 4 and 5). This result could be explained by the role of angiogenesis-related proteins identified in pECM proteomics (PDGFB, VWF, AGT, EGFL6, EGFL7, TYPM, HSPG2 TGFβ1, HIF1A, ITGAV, ITGB3, CDH5) and a series of angiogenesis factors (such as VEGF, WNT5A, AKT1, CD248, SPON2, etc.) in previous studies [38–40]. We also demonstrated that pECM grafts showed denser extracellular matrix remodeling after transplantation (Fig. 9), which may be related to the presence of tissue remodeling-related proteins (MMP19, TIMP3, IGF2) and abundant extracellular matrix proteins (collagen, fibronectin, laminin) in pECM. However, the insufficient mechanical property of pECM limited its application in mechanical loading tissue repair. The hybrid scaffolds had complementary advantages of synthetic polymer and dECM materials. Co-electrospinning was a typical fabrication method and provided the ability to manufacture multi-scale fibrous scaffolds. Compared with PCL scaffolds, the hybrid scaffolds composed of PCL microfiber and epECM nanofiber not only owned enough mechanical strength, but also provided abundant of bioactive modification sites (Fig. 1). Heparinization of pECM also improved antithrombotic properties (Fig. 2), providing the capability and flexibility for various bioactive factors modification [41,42].

Immune cells including macrophages play a vital role in tissue regeneration. Macrophage polarization can regulate tissue vascular regeneration [43]. Anti-inflammatory macrophages have been proved to promote endothelialization, and vascularization, and prevent IL-1β-IL-6-induced macrophages activation and polarization [44–46]. Our results illustrated that incorporating pECM can enhance the immune regulation ability of PCL scaffolds, and the IL-4 modification further advanced the anti-inflammatory polarized efficiency of macrophages of pECM, including the expression of marker proteins, morphological changes, and factors secretion (Figs. 3 and 6). IL-4 had been proved to be contributable to vascular stabilization at the anastomosis [19]. IL-10 was found to augment the expression of Fascin-1 and β-catenin on ECs which supported cell migration, adhesion, angiogenesis and increased VEGF expression [45,47]. In addition, IL-10 also increased the survival and migration of human endothelial progenitor cells [48]. Conversely, TNF-α had been observed to induce VSMCs mineralization and osteoblastic differentiation in vitro [49]. IL-1β triggered the secretion of various pro-inflammatory cytokines related to the level of stenosis and athetosis [50,51]. Consistent with the results mentioned above, the PCL-epECM/H-IL-4 scaffolds increased the expression of anti-inflammatory cytokines (IL-4 and IL-10), and attenuated the expression of pro-inflammatory cytokines (IL-1β and TNF-α) (Fig. 3). Similarly, In PCL-epECM/H-IL-4 group, the polarized macrophages further promoted the migration and maturation of VSMCs in vitro (Fig. 5) and in vivo (Fig. 8), which was also verified by Takeda et al. study [52]. Moreover, the ECM (including collagen, elastin, and GAG) secreted by VMSCs was denser and more oriented in PCL-epECM/H-IL-4 group. M2 macrophages can promote ECM remodeling [53,54]. Macrophage depletion also results in weak VSMCs regeneration and ECM deposition within the graft wall, indicating the critical role of macrophages in successful graft remodeling [55,56]. Many previous studies mainly focused on directly regulating the behaviors of ECs and VSMCs [57–60], while our findings demonstrated that immunomodulatory activity modification optimized the performance of SDVGs and provided a new approach for the activity modification of hybrid scaffolds for vascular regeneration.

Although our study improved vascular regeneration, some issues need to be solved. For example, the combined modification of multiple factors and accurate biomimetic structure design is worthy of further investigation. The hybrid scaffolds should be evaluated in animal models of aging and vascular disease [61]. The clinical translational potential of hybrid scaffolds needs to be further validated using large animal models.

5. Conclusion

The co-electrospinning technology was used to fabricate biodegradable synthetic polymers microfiber and dECM nanofiber, which endowed the hybrid grafts with fine biological activity and suitable mechanical strength. Through targeted activity modification, the hybrid grafts firstly modulated immune cells and then regulated tissue cells, thereby promoting vascular tissue regeneration. In short, our study provides a new strategy for the preparation and activity modification of vascular scaffolds, and can also be used for other tissue regeneration (nerve, muscle, myocardium, etc.).

Data and materials availability

All data associated with this study are present in the paper or the Supplementary Materials.

Ethics approval and consent to participate

The animal study was approved by the Animal Experiments Ethical Committee of Nankai University and complied with the Guideline for Care and Use of Laboratory Animals. The accreditation number of the laboratory is SYXK(Jin) 2019-0001 promulgated by Tianjin Science and Technology Commission. All studies using human samples were approved by the Donation Service of Tianjin Central Hospital of Gynecology and Obstetrics and Tianjin Central Hospital of Gynecology and Obstetrics’s clinical research ethics committee (approval number2022KY0556), which has regulations consistent with the Helsinki Declaration.

CRediT authorship contribution statement

Siyang Liu: Validation, Formal analysis, Investigation, Writing – original draft, Visualization. Liying Yao: Validation, Investigation, Writing – original draft. Yumeng Wang: Investigation, Software,
Declaration of competing interest

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