Neutrophil membrane-camouflaged nanoparticles alleviate inflammation and promote angiogenesis in ischemic myocardial injury

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A R T I C L E  I N F O

Keywords:
Biomimetic nanoparticles
Interleukin-5
Eosinophils
Angiogenesis
Myocardial infarction

A B S T R A C T

Acute myocardial infarction (AMI) induces a sterile inflammatory response, leading to cardiomyocyte damage and adverse cardiac remodeling. Interleukin-5 (IL-5) plays an essential role in developing eosinophils (EOS), which are beneficial for the resolution of inflammation. Furthermore, the proangiogenic properties of IL-5 also contribute to tissue healing following injury. Therefore, targeted delivery of IL-5 is an innovative therapeutic approach for treating AMI. It has been shown that conventional IL-5 delivery can result in undesirable adverse effects and potential drug overdose. In this study, we successfully synthesized a biomimetic IL-5 nanoparticle by camouflaging the IL-5 nanoparticle in a neutrophilic membrane. The administration of neutrophil membrane-camouflaged nanoparticles (NM-NP\textsubscript{IL-5}) in the in vivo model showed that these nanoparticles promoted EOS accumulation and angiogenesis in the infarcted myocardium, thereby limiting adverse cardiac remodeling after AMI. Our results also demonstrated that the NM-NP\textsubscript{IL-5} could serve as neutrophil “decoys” to adsorb and neutralize the elevated neutrophil-related cytokines in the injured heart by inheriting multiple receptors from their “parent” neutrophils. Finally, the targeted delivery of NM-NP\textsubscript{IL-5} protected the cardiomyocytes from excessive inflammatory-induced apoptosis and maintained cardiac function. Our findings provided a promising cardiac detoxification agent for acute cardiac injury.

1. Introduction

Acute myocardial infarction (AMI) is one of the leading causes of death worldwide. Medical and interventional therapies for AMI have been well established in recent decades. However, the long-term survival rate of patients who suffer AMI remains unsatisfactory due to ventricular remodeling and end-stage heart failure \cite{1,2}. The persistent loss of cardiomyocytes (CMs) in the infarcted area is the primary cause, which leads to irreversible damage to the myocardium or even sudden cardiac death \cite{3}. Stem cell transplantation is one of the regenerative strategies that have been blossomed in recent years to replenish the loss of CMs \cite{4,5}. However, low survival and retention rates in infarcted myocardium severely limit their therapeutic efficiency. Direct conversion of noncardiomyocytes into CMs seems like a feasible solution for overcoming these challenges. Nevertheless, direct reprogramming of noncardiomyocytes into cardiomyocyte-like cells (iCMs) requires complex reprogramming techniques and with low efficiency \cite{6,7}, especially \textit{in vivo} \cite{8,9}. It is more critical that these reprogrammed cells hold only minor components of the natural CMs, unable to compensate for the loss of CMs completely \cite{10}. Therefore, it is more urgent to focus on other regeneration strategies, such as angiogenesis, for which endothelial cells have shown a more aggressive division and differentiation capacity.

Interleukin (IL) – 5 was once thought to be a T-cell replacement factor and B-cell growth factor II \cite{11}. Recent studies have found that IL-5 can dramatically increase the proliferation, migration, and tube formation of human umbilical vein endothelial cells (HUVECs) \cite{12},
indicating that it may act as an angiogenesis activator during injury. Additionally, Eosinophils (EOS) have been shown to promote tissue repair by secreting various chemokines, cytokines, growth factors, and enzymes [13], as well as extensive communication with a wide range of cells [14]. Recently, it has been demonstrated that EOS also exerts cardioprotective properties in infarcted hearts [15,16]. Since IL-5 plays a critical role in the formation, activation, and survival of EOS [17], we propose that IL-5 can be a promising cardiac detoxification agent during the inflammatory phase in post-MI events.

However, the delivery approach of the cytokines remains to be fine-tuned. Cytokines act on a wide variety of receptors, potentially leading to unwanted adverse effects and posing safety risks if overdosed [18]. Additionally, the difficulty associated with preserving the bioactivity of cytokines in vivo also hampers their application [19]. Therefore, a precise delivery system is essential for the therapeutic application of cytokine.

The advances in drug delivery systems (DDSs) have presented new possibilities for improving the accuracy of drug delivery [20,21]. In particular, cell membrane coating nanotechnology is an emerging nanoparticle delivery technique that bestows nanoparticles with enhanced biointerfacing capabilities by directly coating the nanoparticles with naturally derived cell membranes [22,23]. Therefore, these nanoparticles not only inherit the antigenic character of the source cells but also interact with specific cells while avoiding clearance by the monocyte/macrophage system. Neutrophils are the most abundant immune cells in the peripheral blood. They are also the first cells to infiltrate the infarcted myocardial region and subsequently interact with endothelial cells through increased adhesion molecules [24]. Therefore, encasing therapeutic nanoparticles in the naturally derived neutrophil membrane can prolong the life of nanoparticles in peripheral blood and extend the interaction between nanoparticles and injured tissue. In this study, we developed a neutrophil decoy, which is composed of polyethylene glycol-polyactic acid (PLGA) polymeric cores loaded with IL-5 and naturally derived and activated neutrophil membrane coating (NM-NP_{IL-5}) (Scheme 1). We further investigated its effects on angiogenesis and immunosuppression for cardiac recovery both in vitro and in vivo.

2. Methods

2.1. Preparation and characterization of NM-NP_{IL-5}

The NM-NP_{IL-5} was prepared by the double-emulsion solvent evaporation [25]. Briefly, 1% of IL-5 solution was made by dissolving 100 μg of IL-5 in 100 μl phosphate buffer solution (PBS). 5% of poly(lactic-co-glycolic) acid (PLGA) was made by dissolving 100 mg of carboxyl-terminated 50:50 poly(lactic-co-glycolic) acid (PLGA) into 2 ml of dichloromethane. The IL-5 solution was then added to the oil phase

Scheme 1. Schematic illustration of preparation of NM-NP_{IL-5} for cardiac healing.
and sonicated for 3 min to form the primary emulsion. Next, the primary emulsion was mixed with 4 ml of 0.5% polyvinyl alcohol (PVA) and sonicated for 3 min to create the secondary emulsion. The mixture was stirred for 2–3 h to evaporate the organic solvent. Nanoparticles were collected via centrifugation at 12,000 rpm, 4 °C, 20 min. The equation detected the encapsulation efficiency percentage (EE %) of the drug: (EE %) = [(W\text{initial drug} - W\text{free drug}) / W\text{initial drug}] × 100%.

For the NM-NPs construction, the above neutrophil membrane was mixed with nanoparticles in a 2:1 membrane-protein-core-weight ratio and subsequently sonicated for 2–3 min on ice. The mixture was centrifuged for 50 min at 14,000 rpm, 4 °C, to remove the excess membrane. Cy5.5 was loaded into NPs at weight ratios of 0.1% and 1%, respectively, for in vitro and in vivo experiments.

The size and zeta potential were detected by Mastersizer 3000 (Malvern Pananalytical, Malvern, UK). The morphology was captured using JEOL 2010 transmission electron microscopy (TEM). For in vitro release investigation, NPs and NM-NPs were added to PBS and incubated for 1, 4, 8, 24, 48, 120 h at 37 °C. Then, the NPs were removed by centrifugation. The concentrations of IL-5 were determined using an enzyme-linked immunosorbent assay (ELISA) as directed by the manufacturer (Elabscience, Houston, Texas, USA).

2.2. Identification of membrane-associated protein

As previously described, Coomassie blue staining and Western blotting were performed to confirm the presence of neutrophil membrane-associated proteins [26]. Briefly, the neutrophil lysate (NL), neutrophil vesicles (NVs), and NM-NPs were lysed on ice for 10 min in lysis buffer (Solarbio, Beijing, China). The lysates were centrifuged at 12,000×g for 10 min, and the supernatant was collected. After that, the SDS loading buffer was mixed with the supernatant and boiled for 5 min. All samples were loaded into each well at 20 μg of protein. For complete imaging, the gelatin was stained using Coomassie blue staining solution (Beyotime, Haimen, China). For Western blotting, all proteins were transferred to polyvinylidene fluoride membranes (Millipore, MA, USA) and blocked with 5% nonfat milk for 2 h at 37 °C. Next, the primary antibodies against TNFα-R, LFA-1, integrin β2, PSGL-1, IL1R2, CXCXR2, CCR1 (Abcam, Cambridge, UK), and β-actin (Proteintech, Wuhan, China) were incubated with the bands overnight at 4 °C before being incubated with secondary antibodies. Finally, the protein bands were visualized using AI 600 software (GE, Healthcare, Chicago, USA).

2.3. In vitro binding assay

Human umbilical vein endothelial cells (HUVECs) were cultured in an ECM medium for 6 h with 100 ng/ml of necrosis factor-α. The expression of ICAM-1 was determined by immunofluorescence assay. For nanoparticle binding, Cy5.5-labeled NPs or NM-NPs were added to PBS and incubated for 1, 4, 8, 24, 48 h at 37 °C. Then, the NPs were removed by centrifugation. The concentrations of IL-5 were determined using an enzyme-linked immunosorbent assay (ELISA) as directed by the manufacturer (Elabscience, Houston, Texas, USA).

2.4. Mouse model of MI

C57BL/6 N mice (8 weeks old, male) were purchased from Weitong Lihua Experimental Animal Technology Co. Ltd (Beijing, China). The AMI animal model was created as described previously [27]. All mice operations complied with the Institutional Animal Care and Use Committee of Zhengzhou University. Briefly, mice were anesthetized using ketamine/xylazine (80 mg/kg ketamine and 4 mg/kg xylazine, i.p.), and a small-animal ventilator (Harvard Apparatus, MA, USA) was used for ventilation. The anterior descending coronary artery was then ligated with a 7-0 Vicryl suture. After that, the chest was closed, and the mice were kept on ventilating for 10 min. Following surgery, all mice were randomized into three groups: PBS (300 μl), NPsL (300 μl), and NM-NPsL (300 μl) (intravenous injection). The total dosage of IL-5 was administrated at 100μg/kg, as described in detail by Xu et al. [16]. Mice were sacrificed on days 3 and 7 for short-term experiments and day 28 for long-term experiments.

2.5. Pharmacokinetics and clearance of NPs in vivo

To evaluate the pharmacokinetics of NPs in vivo, C57BL/6 N mice (8 weeks old, male) were intravenously administered with 2 mg Cy5.5-labeled NPs and NM-NPs. Blood samples were collected at 1, 3, 6, 12, 24, 48, and 72 h respectively. The fluorescence intensity was detected using a plate reader.

For the in vivo clearance detection, Cy5.5-labeled NPs were injected into mice via the tail vein after MI surgery. The immune cells were then extracted from both blood using red lysis buffer and other organs (spleen, liver, heart) using enzymatic digestion. The immune cell pellets were incubated with violetFluor™ 450-conjugated anti-CD45 antibody, and the pellets were resuspended in FACS buffer and analyzed using flow cytometry.

2.6. In vivo targeting ability assay

For the in vivo targeting ability of nanoparticles, mice were injected with equal volumes of PBS, Cy5.5-labeled NPsL and Cy5.5-labeled NM-NPsL via the tail vein after MI surgery. The images were captured using an IVIS Spectrum imaging system (Caliper LifeSciences, USA) at 6 h, 1 d, 3 d, and 7 d respectively. Excitation and emission were respectively, set at 673 nm and 707 nm.

To further investigate the distribution of nanoparticles in vivo, major organs (heart, liver, spleen, lung, kidney, and brain) were collected under euthanasia conditions. Each organ’s fluorescence signal was quantified using the IVIS Spectrum imaging system.

2.7. Cardiac function assessment

Mice were anesthetized with isoflurane, and transthoracic echocardiography was performed on 0 d, 7 d, and 28 d, respectively, to assess cardiac function. Each heart was evaluated by the 2D M-mode, and the cardiac parameters were analyzed under double-blind conditions. Mice with significant outliers of left ventricular ejection fraction (LVEF%) and fraction shorting (LVFS%) on 7 days were excluded from the subsequent studies.

2.8. Immunohistochemistry and immunofluorescent staining

For immunohistochemistry, paraffin-embedded sections were boiled in citrate solution before being blocked with endogenous hydroperoxide. Next, 0.3% Triton X-100 (containing goat serum) was used to permeabilize and block the sections for 1 h. The primary anti-Siglec-F antibody was then incubated with sections at 4 °C overnight. The secondary antibody conjugated with horseradish peroxidase was incubated with the sections for 1 h at room temperature. Siglec-F® cells were counted under different random infarct/injury fields.

For immunofluorescence, tissue sections were washed with PBS for 10 min and blocked with blocking solution for 1 h at room temperature. Then, the sections were incubated overnight at 4 °C with the primary antibodies (α-SA, CD31, CD68, CD206, and MPO). Next, incubated fluorescent secondary antibodies with sections for 2 h at room temperature. Finally, the slides were counterstained with DAPI for 5 min and coverslipped. The images were taken by a confocal microscope. Data were analyzed by blind.
2.9. Histological analysis

Masson’s trichrome staining was used to evaluate the infarct size and wall thickness. The sections were stained with Masson’s trichrome according to the manufacturer’s instructions. Infrac size was calculated using ImageJ software. 2,3,5-triphenyl tetrazolium chloride (TTC) staining was used to evaluate the injury in the acute phase. Briefly, the hearts were harvested and frozen at $-80 \, ^\circ \text{C}$ for 1 h to cut into 5 slices below the ligature. The slices were then incubated with TTC solution at $37 \, ^\circ \text{C}$ for 30 min without light and fixed with 4% paraformaldehyde for 4 h at room temperature. The images were taken using a digital camera, and the infract size was analyzed blind.

2.10. Statistical analysis

All data were present as mean ± SD. GraphPad Prism 8.0 was used to analyze the data. The unpaired two-tailed unpaired Student’s t-test was used for a single comparison. One-way ANOVA followed by Tukey test or two-way ANOVA followed by Bonferroni post hoc test was used for multiple group comparisons. $P$ value $< 0.05$ was considered statistically significant.

3. Results

3.1. Preparation and characterization of neutrophil membrane-enveloped IL-5 nanoparticles (NM-NP$_{IL-5}$)

NM-NP$_{IL-5}$ was synthesized by a stepwise process. First, neutrophils were isolated from the blood using a Percoll gradient separation method according to the previously described [28]. The neutrophil-derived membrane vesicles (NMVs) were then obtained by a series of centrifugation from the cell homogenate. Then, IL-5 was loaded into PLGA NPs using the emulsion/solvent evaporation method [29]. Finally, the neutrophil membrane was coated on the surface of NP$_{IL-5}$ by mixing them. The drug-loading capacity and encapsulation efficacy of NM-NP$_{IL-5}$ were calculated to be 0.47% and 53.9%, respectively. TEM showed a neutrophil membrane layer on the surface of the NM-NP$_{IL-5}$, which was not observed on the NP$_{IL-5}$ (Fig. 1A). Due to the membrane enveloping, the size of NM-NP$_{IL-5}$ increased by about 20 nm compared with that of NP$_{IL-5}$. Besides, NM-NP$_{IL-5}$ possessed less negative zeta potential than NP$_{IL-5}$, again proving that a biomimetic surface charge was formed (Fig. 1B). In terms of drug release, NM-NP$_{IL-5}$ was released gradually over time (Fig. 1C). Stability of NPs was also detected under different pH and fetal bovine serum (FBS) concentrations, as shown in Fig. 1D and E. The diameter of NM-NP$_{IL-5}$ exhibited a mild increase over time. As a result of our research, we were able to coat neutrophil membranes on the surface of PLGA NPs, and the engineered delivery system was more stable and had sustained release properties.

To determine whether the proteins on the neutrophil membrane had

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**Fig. 1.** Characterization of NM-NP$_{IL-5}$. (A) Transmission electron microscope image of bare PLGA, NP$_{IL-5}$, and NM-NP$_{IL-5}$. Scale bar = 100 nm. (B-C) Diameter and Zeta potential of NP, NP$_{IL-5}$, and NM-NP$_{IL-5}$. n = 3. (C) Release profile of IL-5 in PBS over time. (D) Stability of NM-NP$_{IL-5}$ over time in pH = 6.4 and 7.4. n = 3. (E) Stability of NM-NP$_{IL-5}$ in PBS with or without 20% FBS over time. n = 3. (F) Total protein content visualization of neutrophil lysates (NL), neutrophil membrane-derived vesicles (NV), and neutrophil-NP (NM-NP) stained with Coomassie Brilliant Blue. (G) Western-blot identification of neutrophil membrane makers (TNF-αR, LFA-1, integrin β1, PSGL-1, IL1R2, CXCRC2, and CCR1) in neutrophil lysates without membrane proteins (NLWNM), NV and NM-NP. (H) Uptake of NP or NM-NP by RAW 264.7 cells over time measured by flow cytometry. All data are shown as the means ± SD. Statistical analyses were performed by Student’s t-test.
been successfully translocated to the surface of PLGA NPs, Coomassie blue staining was used for protein analysis. The results showed that NM-NP\textsubscript{IL-5} and neutrophil membrane vesicles had a similar total protein profile, indicating that most proteins of the neutrophil membrane were translocated to the surface of PLGA NPs, as shown in Fig. 1F. According to previous studies, neutrophil primarily interacts with inflamed endothelium via various adhesion molecules such as selectins and integrins [30]. In order to confirm whether these molecules were highly preserved, a Western blotting was performed. Several representative molecules (TNFα-R, LFA-1, integrin β1, PSGL-1, IL1R2, CXCR2, CCR1) were all detected on NM-NPs and neutrophil lysates without membrane proteins (Fig. 1G). Furthermore, to demonstrate the advantage of naturally derived neutrophil membrane coating in NM-NPs, we incubated both NM-NPs and NPs with RAW 264.7 macrophages for varying lengths of time (2, 4, 6, 12, 24 h). Flow cytometry analysis was used to evaluate the retention rate. The results showed that the biomimetic membrane of neutrophils significantly reduced macrophage clearance, indicating that a longer circulation time could be achieved \textit{in vivo} (Fig. 1H). These findings demonstrate that the neutrophil membrane–coated IL-5 nanoparticles were successfully constructed and have the capacity to target and release.

3.2. \textit{In vitro} targeting ability of NM-NP\textsubscript{IL-5}

It is well known that neutrophils are activated and gradually roll along the internal wall of blood vessels through the binding between ligands and receptors during AMI [31]. Here, before determining the binding of NPs to the inflamed endothelium, we first investigated the potential cytotoxicity of NM-NP\textsubscript{IL-5}. As shown in Fig. 2A and Fig. S1A, the activities of HUVECs and CMs were not adversely affected by 200 mg/L of NM-NP\textsubscript{IL-5}, indicating that the concentration of NPs used in all subsequent tests is safe for cells. We also used immunofluorescence to confirm that the expression of ICAM-1, an integrin ligand, was upregulated in HUVECs after TNF-α (100 ng/ml) stimulation (Fig. 2B and C). Then, NP\textsubscript{IL-5} and NM-NP\textsubscript{IL-5} (cy5.5 labeled) were incubated with TNF-α-stimulated HUVECs. It is noticed that NM-NP\textsubscript{IL-5} had a greater affinity to TNF-α-stimulated HUVECs than NP\textsubscript{IL-5}, as evidenced by immunofluorescence and flow cytometry analyses. However, NP\textsubscript{IL-5} and NM-NP\textsubscript{IL-5} failed to interact with HUVECs in the absence of TNF-α stimulation. Considering the utility of chemotaxis between NM-NP\textsubscript{IL-5} and inflamed HUVECs, which is likely mediated by the interaction of neutrophil membrane proteins (NMP) and ICAM-1. To further prove the role of ICAM-1 in chemotaxis, HUVECs were pretreated with anti-ICAM-1 antibody and were used as a control group. The results demonstrated that blocking ICAM-1 indeed reduced the utility of

![Fig. 2. Specific targeting ability of NM-NP\textsubscript{IL-5} \textit{in vitro}. (A) CCK-8 detection of cell activity at different concentrations of NM-NP\textsubscript{IL-5}. n = 5. (B–C) Representative images and quantification of ICAM-1 expression. n = 3. (D) Representative images of HUVECs incubated with NP\textsubscript{IL-5}, NM-NP\textsubscript{IL-5}, or HUVECs preblocked by anti-ICAM-1 antibody under 100 ng/ml TNF-α or not. (E) Flow cytometry analysis of binding capacity. n = 3. (F) Diagram of the Transwell system. (G) Fluorescence intensity of lower chamber with or without TNF-α stimulation after incubation with NM-NP\textsubscript{IL-5}. (H) Quantification of NP\textsubscript{IL-5} and NM-NP\textsubscript{IL-5} in the lower chamber with TNF-α treatment over time. n = 3. (I) Fluorescence intensity of lower chamber when incubated with Dynasore or Bafilomycin under TNF-α stimulation. n = 3. *P < 0.05 compared with the PBS group. All data are shown as the means ± SD. The comparison between the two groups was performed by Student’s t-test. Comparisons among multiple groups were performed with One-way or Two-way ANOVA.]{fig2.png}
chemotaxis (Fig. 2D and E). These results revealed that the NMP inherited from the neutrophil surface can facilitate adherence to inflammatory endothelial cells in vitro.

To further investigate whether our nanoplatform could cross the endothelial barrier and the underlying molecular mechanisms, a transwell system was established with HUVECs seeded into a 0.4 μm cell insert (Fig. 2F). After HUVECs reached 100% confluency, 100 ng/ml TNF-α was added into the upper chamber medium but not the lower chamber to imitate an in vivo inflammatory situation. Results showed that TNF-α treatment increased fluorescence intensity in the lower chamber (Fig. 2G), suggesting that inflamed environments enhanced the ability of NM-NP_{IL-5} to cross the endothelial barrier. Meanwhile, Fluorescence-labeled NPs were added to the upper chamber for different periods, and the intensity of the fluorescence in the lower chamber was measured to quantify nanoparticles crossing the endothelium barrier. As the results showed, fluorescence-labeled NM-NP_{IL-5} in the lower chamber medium was relatively higher than the bare NP_{IL-5} group with TNF-α stimulation (Fig. 2H), indicating that the activated neutrophil membrane can facilitate the endothelial barrier crossing of NM-NP_{IL-5}.

However, it is uncertain whether the crossing capability was via the enhanced permeability and retention (EPR) effect or increased transcytosis under inflamed conditions. To further explore the exact mechanism, we incubated HUVECs with NM-NP_{IL-5} in the presence of either an endocytosis inhibitor (Dynasore, 50 μM, 1 h) or an exocytosis inhibitor (Bafilomycin A1, 100 μM, 6 h). The fluorescence intensity value showed that neither Dynasore nor Bafilomycin A1 had a significant impact on the number of nanoparticles crossing the endothelial barrier compared with the control group (Fig. 2I), which revealed that transcytosis was not the primary way by which NM-NP_{IL-5} crossed the endothelial layer under inflamed conditions. Then enhanced endothelial permeability may be the critical component for NM-NP_{IL-5} penetrating the endothelial barrier. To confirm this proposal, a fluorescence-labeled 70 kDa dextran was added under normal or inflamed conditions in the upper chamber to evaluate the endothelial permeability. As the results showed, TNF-α treatment caused a significant increase in the intensity of fluorescence in the lower chamber (Fig. S1B), indicating the leak of dextran into the lower chamber and hence increased endothelial permeability under inflamed conditions. Taken together, NM-NP_{IL-5} has the potential to not only adhere to inflamed endothelial cells but also to cross the endothelial barrier, which might be mediated by migration between endothelial cells rather than transcytosis.

3.3. NM-NP_{IL-5} could promote angiogenic responses and adsorption of inflammatory factors and chemokines in vitro

To investigate the angiogenic responses of NM-NP_{IL-5} in vitro, the migration, invasion, and tube formation of HUVECs were assessed using wound-healing assay, transwell migration, and tube formation assays, respectively. We found that NM-NP_{IL-5} significantly increased the wound closure rate, invasiveness, and colony tube length of HUVECs compared with the control group (Fig. 3A–D). These in vitro findings suggested that NM-NP_{IL-5} can promote the angiogenic responses of HUVECs, implying...
that our engineered system may benefit heart healing after injury via angiogenesis, even in vivo.

According to previous studies, the neutrophile membrane vesicles (NMVs) obtained from activated and purified peripheral blood neutrophils can act as a cytokine decoy to neutralize multiple inflammatory factors and chemokines, thereby interrupting the neutrophil infiltration and cell activation [32,33]. Several cytokines have been identified as mediating neutrophil recruitment and infiltration under chronic inflammatory conditions, including TNF-α, IL-1β, CINC-2, and MIP-1α [34, 35]. We next investigated whether NM-NPs could adsorb inflammatory factors and chemokines in vitro via the inherited receptors from their “parental” activated neutrophils by ELISA (Fig. 3E). As shown in Fig. 3F, NM-NPs markedly adsorbed these cytokines (TNF-α, IL-1β, CINC-2, and MIP-1α) in a concentration-dependent manner. The half-maximal

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Fig. 4. In vivo targeting capacity of NM-NP<sub>IL-5</sub> in mice model of MI. (A) Short-term and long-term experimental timelines. (B) The mortality rates of different groups. (C) Circulation profiles of NP<sub>IL-5</sub> and NM-NP<sub>IL-5</sub> in mice after intravenous injection. n = 3. (D) Flow cytometry measured the uptake of NP<sub>IL-5</sub> and NM-NP<sub>IL-5</sub> by CD45<sup>+</sup> leukocytes in the liver, spleen, blood, and heart. n = 3. (E) Representative in vivo images of MI mice at various intervals after intravenous injection of NP<sub>IL-5</sub>@cy5.5 and NM-NP<sub>IL-5</sub>@cy5.5. (F) Quantifying fluorescent intensity in hearts at different time points following intravenous injection of PBS, NP<sub>IL-5</sub>, and NM-NP<sub>IL-5</sub>. n = 3. (G) Ex vivo fluorescent images of major organs at 6 h after intravenous injection. (H) Quantification of fluorescent intensity in major organs. n = 3. *P < 0.05 compared with the NP<sub>IL-5</sub> group. All data are shown as the means ± SD. Statistical analyses were performed by One-way or Two-way ANOVA.
inhibitory concentrations (IC50) of TNF-α (8 ng/ml from the initial concentration) and IL-1β (10 ng/ml from the initial concentration) were 136.2 and 234.7 μg/ml, respectively. The IC50 values of CINC-2 (2 ng/ml from the initial concentration) and MIP-1α (500 pg/ml from the initial concentration) were 936.9 and 563.7 μg/ml, respectively. These data demonstrated that NM-NPIL5 could adsorb cytokines by neutrophil membrane coating.

3.4. Targeting profile of NM-NPIL5 in AMI mice

Our in vitro findings support that NM-NPIL5 can target the human-induced inflammatory endothelial cells and may benefit healing through angiogenesis in an ischemic heart. Thus, to further confirm the effects of NM-NPIL5 in vivo, an AMI model of mice was established by experienced professionals. Mice were randomized into two groups for the in vivo experiments: short-term and long-term groups, as described in Fig. 4A. The mortality rates of the treatment groups (NPIL5 and NM-NPIL5 groups) after AMI are lower than that of the control group (PBS), indicating that IL-5 can reduce mortality rates in both long and short-term experiments. It is also noted that mice treated with NM-NPIL5 had an even lower mortality rate, indicating that the neutrophil membrane coating on IL-5 nanoparticles exhibited a better targeting ability. However, a slightly increased mortality was observed in long-term experiments.

The circulation time is critical for targeted nanoparticles, and an extended circulation time can promote more nanoparticle recruitment to the target organ [36]. To evaluate the circulation time and clearance rate of NPIL5, the pharmacokinetics evaluation was performed by calculating the fluorescence intensity of fluorescence-labeled nanoparticles at different time points. As shown in Fig. 4C, NM-NPIL5 exhibited a longer circulation time than NPIL5 due to the enveloped biomimetic membrane component. Furthermore, the uptake of nanoparticles in the circulation and main organs by phagocytes was conducted by flow cytometry. The mice were sacrificed 6 h after receiving NPIL5 or NPIL5 intravenously. Phagocytes from the liver, spleen, blood, and heart were collected and incubated with violet-Fluo1450-conjugated anti-CD45 primary antibody for a flow cytometry assay. As shown in Fig. 4D and Fig. S2, internalized nanoparticles by phagocytes were significantly reduced in the presence of biomimetic enveloping, implying that the neutrophil camouflage strategy can significantly reduce the clearance rate of nanoparticles in the circulating system. These findings suggest NM-NPIL5 can be beneficial to the injured heart. To prove that, we assessed the ability of NM-NPIL5 to be recruited to the injured myocardium. The fluorescence intensity in the major organs (hearts, livers, spleens, kidneys, and brains) of mice was visualized and calculated using the IVIS system at set time points. The findings revealed that NM-NPIL5 injection significantly higher accumulation in the heart than NPIL5 treatment, which was facilitated by neutrophil chemotaxis targeting. A large number of nanoparticles were also observed to accumulate in the liver and lung as well (Fig. 4E-H). Overall, these data revealed that NM-NPIL5 had a long blood circulation time and a higher accumulation rate in the injured heart, which benefits heart repair.

3.5. NM-NPIL5 promotes EOS in the infarcted myocardium while inhibiting cardiomyocyte apoptosis

This is in contrast to most targeted delivery systems, which frequently experience off-target issues, resulting in the ‘cargos’ being released into the blood circulation or unwanted organs and adverse effects [37]. Our neutrophil camouflaged system has the potential to optimize these drawbacks. Because IL-5 is required for EOS maturation, activation, and survival [38], EOS plays a critical role in the ischemic heart by promoting the macrophage signal transducer. Even the released portion in circulation can benefit the immune regulation of AMI by increasing the number of EOS recruited to an injured heart. Therefore, we investigated the effects of NPIL5 and NM-NPIL5 on the number of EOS in peripheral blood and myocardium. We discovered that both NPIL5 and NM-NPIL5 could increase the number of EOS in the peripheral blood, with NM-NPIL5 exhibiting a more significant effect (Fig. 5A). Furthermore, Western blotting analysis revealed that NM-NPIL5 could deliver more IL-5 to the injured myocardium (Fig. 5B). The EOS count in infarcted myocardium was also revealed by H&E staining and anti-Siglec-F anti-body staining of heart sections on day 3 post-AMI. As shown in Fig. 5C-E, NM-NPIL5 treatment significantly increased EOS recruitment in infarcted myocardium. Although we noted that NPIL5 could increase the number of EOS in peripheral blood and infarcted myocardium, the effect was weaker, likely due to the absence of biomimetic membrane protection.

To determine whether enhanced recruitment of EOS in the myocardium could alleviate cardiac injury, the early infarct size was assessed by TTC staining. Compared with the group that received PBS, data showed that the infarcted size after AMI was significantly reduced with NM-NPIL5 and NPIL5 treatment (Fig. 5F and G), and the NM-NPIL5 group showed a relative smaller infarct size than the NPIL5 group. TUNEL staining, on the other hand, was used to identify apoptotic cells in the early infarcted myocardium. As expected, the NM-NPIL5 group held the fewest apoptotic positive cells (Fig. 5H and Fig. S3). Bcl-2 and Bax are antagonists in apoptosis. This process is inhibited by Bcl-2, whereas it is promoted by Bax [39]. The Western blot results showed that NM-NPIL5 could increase the Bcl-2/Bax ratio, which was significantly reduced after AMI (Fig. S1 and K). We also evaluated the other two involved proteins in apoptosis, cleaved PARP, and caspase-3 proteins. As shown in Fig. 5I, J, and L, NM-NPIL5 treatment inhibited cleaved PARP and caspase-3 protein levels.

To assess cardiac function in the early stage, we performed echocardiography seven days post-AMI. As expected, the data showed that mice without treatment had significantly decreased left ventricular ejection fraction (LVEF%) and fractional shortening (LVFS%). LVEF% and LVFS% were significantly restored when treated with IL-5 nanoparticles with the fact that NM-NPIL5 exhibited a superior effect than NPIL5 due to the neutrophil membrane camouflage (Fig. 5M and N). Taken together, NM-NPIL5 treatment can improve early-stage cardiac function induced by AMI via enhanced EOS recruitment and inhibited apoptosis.

3.6. NM-NPIL5 administration reduces neutrophile infiltration while enhances cardiac M2-like polarization of macrophages post-AMI

Compared with targeting a specific cytokine using a single antibody, NM-NPIL5 could act as a cytokine decoy inhibiting the recruitment and activation of neutrophils by their inherited multiple membrane receptors. Thus, the infiltration of neutrophils in the myocardium was analyzed by immunofluorescence in mice. Three days after treatment, the NM-NPIL5 group demonstrated lower infiltration of neutrophils in the myocardium than other groups (Fig. 6A and Fig. S4A).

Alternatively activated macrophages have been shown to have cardioprotective effects after AMI by inhibiting inflammation and regulating fibroblast activation [40]. According to previous studies, EOS can maintain alternatively activated macrophages in adipose tissues [41]. The beneficial properties of NM-NPIL5 may be mediated by sloping alternatively activated macrophage accumulation in the injured myocardium following increased EOS recruitment. To verify the proposed hypothesis, we checked the number of CD68+CD206+ macrophages in the heart seven days after AMI. Immunofluorescent staining detection revealed that more CD68+CD206+ positive macrophages were observed in NPIL5 treated mice (Fig. 5B and C). Consistent with the above findings, the mRNA levels of M2-like macrophage makers (Arg1 and Mrc1) increased after NM-NPIL5 treatment (Fig. 6D-G, and Table S1). Similar results were also confirmed by Western blotting analysis (Fig. 6H-J, and Fig. S4B).

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3.7. NM-NP \textsubscript{IL-5} increases angiogenesis post-AMI

Previous research found that IL-5 increased the angiogenic properties of HUVECs via the eNOS pathway \cite{12}. To test whether NM-NP \textsubscript{IL-5} can promote angiogenesis in the infarcted myocardium, we assessed the density of vessels by immunofluorescent staining using an anti-CD31 antibody. As shown in Fig. 7A and B, the NM-NP \textsubscript{IL-5} administration significantly promoted the vessel formation in the infarct border zone. To further investigate the mechanisms underlying the proangiogenic effects, we analyzed the signal pathway, including AKT and ERK1/2, using Western blotting analysis. Treatment with NM-NP \textsubscript{IL-5} significantly increased the phosphorylation of AKT and ERK1/2 (Fig. 7C–E). These results prove that the beneficial effects of NM-NP \textsubscript{IL-5} in infarcted myocardium are also mediated by its proangiogenic capacity.

3.8. NM-NP \textsubscript{IL-5} improves cardiac function in a long-term MI model

Cardiac remodeling is a long-term irreversible consequence after a
cardiac attack. The changes in the size and shape of the heart during remodeling result in cardiac dysfunction, eventually, heart failure [1]. To investigate the long-term therapeutic effect of NM-NP\textsubscript{IL-5}, we evaluated the cardiac function on Day 28 after AMI surgery. From Fig. 8A–C, we can observe a significant amount of viable ventricular tissue in the NM-NP\textsubscript{IL-5} group compared to that in the PBS and NP\textsubscript{IL-5} groups at the end of the study. In addition, transthoracic echocardiography revealed a decrease in LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV), as well as an increase in LVEF% and LVFS%. These results indicated that both NP\textsubscript{IL-5} and NM-NP\textsubscript{IL-5} improved cardiac function and slowed down the cardiac remodeling process (Fig. 8D–H). NM-NP\textsubscript{IL-5} exhibited an even robust effect on preserving cardiac function due to the biomimetic membrane structure. The in vivo biosafety was evaluated using H&E-stained sections of major organs and serum hepatorenal function.

Fig. 6. NM-NP\textsubscript{IL-5} reduces neutrophil infiltration and increases M2-like macrophage accumulation in the myocardium. (A) Infiltration of neutrophils in the infarcted area 3d was detected by myeloperoxidase (MPO). Scale bar = 20 μm. (B–C) Representative immunofluorescent staining and quantification of CD206\textsuperscript{+} macrophage at 7d post-MI. Scale bar = 20 μm n = 5. (D–G) Relative expression of M1 makers and M2 makers at 7d post-MI. n = 5. (H–J) Representative images and quantification of Western blot assay of M1 makers and M2 makers. n = 3. *P < 0.05 compared with the PBS group. **P < 0.05 compared with the NP\textsubscript{IL-5} group. All data are shown as the means ± SD. Statistical analyses were performed by One-way ANOVA.
function analysis for alanine transaminase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), and creatinine (Cr). No discernible changes in toxicity were observed between NP$_{IL-5}$, NM-NP$_{IL-5}$, and PBS administration, indicating that minor safety issues need to be concerned for this nanoparticle (Fig. 8I and Figs. S5A–C).

4. Discussion

Large-scale cardiomyocyte death, activation of the innate immune system, and widespread inflammation are all common pathological features of AMI [42]. Stem cell transplantation is a promising strategy for cardiac regeneration; however, current therapies are restricted by weak interaction between potential therapeutic cells and the injured tissue, which is highly inflammatory, resulting in a low survival rate and differentiation potential [43, 44]. Innovative approaches are needed not only to deliver more “seeds” (therapeutic cells or drugs) to their targets but also to improve the “soil,” the harsh environment in the post-MI heart. IL-5 has been widely investigated in allergic diseases as a member of the β common chain cytokine family [11, 45, 46]. Recent research suggests that IL-5 improves cardiac function after AMI by modulating local inflammatory conditions [16]. This therapeutic effect is mainly achieved by increasing the EOS population in the infarcted myocardium. Moreover, previous studies suggested that IL-5 enhances the angiogenic properties of HUVECs and neovascularization of mice by activating the nitric oxide synthase (NOS) pathway [12]. Thus, IL-5 can function as a viable proangiogenic factor, which appears more effective
in healing damaged hearts than expected in cardiomyocyte regeneration. Given the above factors, we developed a biomimetic drug-loaded nanoparticle that delivered IL-5 precisely into the myocardium after an AMI. Compared with the current targeting system, the NM-NP$_{IL-5}$ developed by our team has demonstrated a superior ability to penetrate vascular endothelial cell barriers, as well as dual efficacy in anti-inflammation and proangiogenesis.

IL-5 was previously thought to be a T-cell replacement factor and a B-cell growth factor II [11, 47]. However, many studies have revealed that IL-5 is also involved in the progression of numerous cardiovascular diseases [16, 48]. IL-5 is significantly higher during the early stages of MI in mice and swine [49]. Recombinant IL-5 administration provided cardioprotective effects after MI by increasing EOS recruitment and angiogenesis. Thus, IL-5 may be a promising candidate for the treatment of AMI. However, the challenges of preserving their bioactivity in vivo, rapid clearance, high dosage, and repeated administration severely limited their utilization. Besides, a low concentration of IL-5 (0.4 ng/ml) stimulation can promote the EOS’s proliferation, differentiation, and survival. Notably, the angiogenesis effects of IL-5 depend upon a concentration of 50–100 ng/ml or higher [12, 16]. For the concerns mentioned above, a biomimetic targeting system was developed by coating the neutrophil membrane on the bare IL-5 nanoparticles to deliver IL-5 into the injured myocardium. In the absence of increased risk associated with a higher drug dosage, targeting IL-5 to the myocardial tissue can maximize post-injury angiogenesis while minimizing costs. Furthermore, a higher level of IL-5 in the myocardium extends the survival time of eosinophils [50], allowing them to play a more significant role in promoting healing. The greatest advantage of the targeting delivery nanoparticle is the dual function while reducing the side effects induced by off-target.

**Fig. 8.** Cardiac repair effects of NM-NP$_{IL-5}$ in vivo. (A) Representative images of Masson’s trichrome-stained sections 28d after treatment. (B–C) Quantification of scar tissue and viable myocardium from Masson’s trichrome images. Scale bar = 1 mm n = 5. (D) Representative M-mode echocardiographic images at 28d post-ML. (E–H) Quantification of LVEF%, LVFS%, LVEDV, and LVESV. n = 5. (I) HE staining of histological sections from major organs 28d after injection. Scale bar = 100 μm *$P < 0.05$ compared with the PBS group. **$P < 0.05$ compared with the NP$_{IL-5}$ group. All data are shown as the means ± SD. Statistical analyses were performed by One-way ANOVA.
The bifunctional nanoparticles are coated with a natural neutrophil membrane, which is activated by inflammatory cytokines and has an inherent chemotaxis ability to accumulate in inflammatory locations. In our \textit{in vitro} binding assay showed that NM-NP\textsubscript{IL-5} could bind to HUVECs under TNF-\textalpha stimulation, which could be blocked or at least partially blocked in the presence of an anti-iCAM-1 antibody. This may suggest that the utility of chemotaxis is likely mediated by the interaction between neutrophile membrane proteins and iCAM-1 in endothelial cells. Similarly, according to the \textit{in vivo} organ targeting assay, membrane-coated nanoparticles exhibited a more robust accumulation in the injured heart than the bare group after intravenous injection 6 h. Furthermore, the mechanisms of NM-NP\textsubscript{IL-5} crossing the endothelial cell barrier were demonstrated mainly through the gap between endothelial cells rather than via a transcellular route. Therefore, NM-NP\textsubscript{IL-5} may be targeted to the infarcted myocardium by its precise chemotaxis and barrier-penetrating ability.

Meanwhile, Wang et al. have demonstrated that neutrophil membrane-camouflaged NPs could be used as ‘decays’ with anti-inflammatory properties after spinal cord injury [32]. Unlike existing targeting and anti-cytokine agents that perform single molecular targeting and inhibit only a narrow range of cytokines, neutrophil-NPs deliver a broad-spectrum, function-driven, and disease-relevant blockade that dampens the inflammation cascade during disease progression. These results are consistent with our detections, in which NM-NP\textsubscript{IL-5} administration decreased the level of a wide variety of proinflammatory cytokines related to neutrophil decays indirectly. In addition, membrane-coated NPs showed longer circulation times than bare NPs, further supporting previous findings [51,52].

In general, the most common methods of drug administration require high dosages and repeated administrations, such as oral and intravenous administration [53,54]. Based on previous reports of the dosage and duration of IL-5 administered to mice, 100 \(\mu\text{g}/\text{kg}\) of recombinant IL-5 was administered daily for three consecutive days. However, a lower dosage of IL-5 was used in our treatment group, yet similar therapeutic effects were achieved. A one-time administration of NM-NP\textsubscript{IL-5} can significantly boost EOS counts in the myocardium and peripheral blood, as well as enhance angiogenesis at the border zone after AMI. Furthermore, it is worth noting that the IL-5-induced EOS effects are achieved through the release of IL-5 in blood circulation, which is then carried to the bone marrow by blood exchange. A further advantage of this dosage was that it was lower than the previous reported, which inhibited cardiomyocyte apoptosis while maintaining left ventricular function. Additionally, our biomimetic targeting system was safe and harmless, with no obvious adverse effects \textit{in vitro} as well as \textit{in vivo}.

5. Conclusion

In summary, our findings demonstrate a well-fabricated biomimetic bifunctional targeting nanoparticle, which promoted inflammation resolution and angiogenesis both \textit{in vitro} and \textit{in vivo}, leading to improved cardiac function post-MI. We unveil the unique and superior ability of neutrophile membranes to serve as targeting and immuno-quivescent envelopes for the delivery of therapeutic drugs to injured lesions.

Ethics approval and consent to participate

The animal study was approved by the Ethical Committee of Zhengzhou University (ZZU-LAC2021083102). All the animals were purchased from Weitong Lihua Experimental Animal Technology Co. Ltd., and the animal certification is No. SCXK (Jing) 2021-0006. All mice operation was compliant with the Institutional Animal Care and Use Committees of Zhengzhou University.

CRediT authorship contribution statement

Dongjian Han: Conceptualization, Methodology, Formal analysis, Investigation, Validation, Visualization, Software, Writing – original draft. Fuhang Wang: Methodology, Software, Validation, Visualization. Zhentao Qiao: Methodology, Validation. Bo Wang: Methodology. Yi Zhang: Software. Qingjiao Jiang: Methodology. Miaomiao Liu: Methodology. Yuansong Zhuang: Methodology, Validation. Quanxu An: Methodology, Visualization. Yan Bai: Software. Jiahong Shangguan: Software. Jining Zhang: Supervision, Writing – review & editing. Gaofeng Liang: Conceptualization, Methodology, Writing – review & editing. Deliang Shen: Conceptualization, Supervision, Methodology, Funding acquisition, Writing – review & editing.

Declaration of competing interest

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

Acknowledgments

This work was financially supported by the National Science Foundation of China (Grant Nos. 81873493 and 82170271 to DL.S.). Henan Thousand Talents Program (Grant Nos. 204200510017 to DL.S.). Thanks for the help of the research and innovation team project of the First Affiliated Hospital of Zhengzhou University.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| AMI | Acute myocardial infarction |
| IL-5 | Interleukin-5 |
| EOS | eosinophil |
| NM-NPs | neutrophil membrane-camouflaged nanoparticles |
| CMs | cardiomyocytes |
| iCMs | cardiomyocyte-like cells |
| HUVECs | human umbilical vein endothelial cells |
| DDs | drug delivery systems |
| PLGA | polyethylene glycol-polyactic acid |
| PBS | phosphate buffer solution |
| PVA | polyvinyl alcohol |
| EE% | efficiency percentage |
| TEM | transmission electron microscopy |
| ELISA | enzyme-linked immunosorbent assay |
| NL | neutrophil lyse |
| NVs | neutrophil vesicles |
| DAPI | 4’,6-diamidino-2-phenylindole |
| LVEF% | left ventricular ejection fraction |
| LVF% | left ventricular fraction shorting |
| TTC | 2,3,5-triphenyl tetrazolium chloride |
| PBS | fetal bovine serum |
| NMP | neutrophil membrane proteins |
| LVEDV | left ventricular end-diastolic volume |
| LVESV | left ventricular end-systolic volume |
| ALT | alanine transaminase |
| AST | aspartate transaminase |
| BUN | blood urea nitrogen |
| Cr | creatinine |
| NOS | oxide synthase |

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.11.016.

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