Published in final edited form as:

Biomaterials. 2019 March ; 197: 244–254. doi:10.1016/j.biomaterials.2019.01.020.

A New Class of Biological Materials: Cell Membrane-Derived Hydrogel Scaffolds

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Abstract

Biological materials are superior to synthetic biomaterials in biocompatibility and active interactions with cells. Here, a new class of biological materials, cell membrane-derived hydrogel scaffolds are reported for harnessing these advantages. To form macroporous scaffolds, vesicles derived from red blood cell membranes (RBCMs) are chemically crosslinked via cryogelation. The RBCM scaffolds with a pore size of around 70 μm are soft and injectable. Highly biocompatible scaffolds are typically made of superhydrophilic polymers and lack the ability to encapsulate and release hydrophobic drugs in a controlled manner. However, hydrophobic molecules can be efficiently encapsulated inside RBCM scaffolds and be sustainedly released. RBCM scaffolds show low neutrophil infiltration after subcutaneous injection in mice, and a

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.biomaterials.2019.01.020>.*****

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

significantly higher number of infiltrated macrophages than methacrylate alginate (MA-alginate) scaffolds. According to gene expression and surface markers, these macrophages have an M2-like phenotype, which is anti-inflammatory and immune suppressive. There are also higher percentages of macrophages presenting immunosuppressive PD-L1 in RBCM-scaffolds than in MA-alginate scaffolds. Interestingly, the concentrations of anti-inflammatory cytokine, IL-10 in both types of scaffolds are higher than those in normal organ tissues. This study sheds light on cell membrane-derived hydrogels, which can actively modulate cells in unique ways unavailable to existing hydrogel scaffolds.

Graphical Abstract

Keywords

Tissue regeneration; regenerative medicine; immune modulation; immunoengineering; drug delivery

1. Introduction

Compared with synthetic biomaterials, biological materials are characterized by their improved biocompatibility, active interactions with cells, and other unique functions. Decellularized tissue scaffolds, which provide architecture, and mechanical and biological cues to seeded and/or recruited cells, have been shown to be superior to current synthetic biomaterials for regenerative medicine [1–4]. There are many other examples of biological materials. Hyaluronic acid, one of the most abundant glycans in animal and human bodies, is widely used for fabricating hydrogel scaffolds and nanoparticles [5–9]. By harnessing the specific interactions of DNA base pairs, DNAs can be programmed to fold into various well-defined three-dimensional structures and respond to external signals [10–12].

Cell membrane-derived nanomaterials are another example, showing the power of biological materials. Red blood cell membrane (RBCM)-coated nanoparticles present “self” protein CD47 and membrane-bound complement regulators, reducing nanoparticle clearance by macrophages in the blood and nanoparticle-induced inflammatory responses to scaffold constructs [13–15]. In another application, the high affinity between cell membranes and pore-forming toxins has been utilized to decrease bacterial virulence [16]. Membrane coatings can also increase nanoparticle targeting thanks to natural receptors on cell membranes [17–21]. More recently, cell membranes with overexpressed immune checkpoint protein, programmed cell death protein 1 (PD-1), have been fabricated into nanovesicles for cancer immunotherapy [22].

Hydrogel scaffolds, especially porous scaffolds, are under extensive study because of their importance in tissue regeneration and immunoengineering [23–30]. Highly biocompatible scaffolds are mostly composed of superhydrophilic and inert materials [15, 31–33]. The lack of biological activity and difficulty in controlled release of hydrophobic drugs have limited the applications of these scaffolds. Inspired by studies using cell membrane-based nanomaterials, we hypothesized that cell membrane-derived scaffolds could enable active biological interactions and the controlled release of hydrophobic drugs in addition to providing necessary biocompatibility. Furthermore, these scaffolds conveniently allow functionalization not only through chemical reactions with the abundant carboxyl groups and primary amines on cell surfaces, but also through physical insertion of bioactive molecules modified with hydrophobic tails into cell membranes [14, 34–37].

In this work, we report a method to create a novel cell membrane-derived hydrogel scaffold and its biological properties (Fig. 1). As a proof of concept, we fabricated scaffolds using RBCMs (RBCM scaffolds), which have a macroporous structure and can encapsulate and sustainably release a hydrophobic model drug. RBCM scaffolds showed good biocompatibility and recruited higher numbers of M2-like macrophages than methacrylate alginate (MA-alginate) scaffolds *in vivo*. Macrophages from RBCM scaffolds also expressed a higher level of immune suppressive programmed death-ligand 1 (PD-L1) than those from MA-alginate scaffolds.

2. Materials & methods

2.1. Preparation of RBCM vesicles

Fresh blood was collected from female C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine, USA) via a sub-mandibular method. All animal procedures were conducted by following protocols approved by the Drexel University Institutional Animal Care and Use Committee in compliance with NIH guidelines. The freshly collected blood was centrifuged at $300 \times g$ for 5 mins. The plasma and white buffy coat were removed. RBCs were then washed with phosphate-buffered saline (PBS) (Lonza) three times and ruptured in 0.2 mM ethylenediaminetetraacetic acid (EDTA, Sigma) water solution. The salt concentration in the solution was adjusted to $1 \times \text{PBS}$ using $10 \times \text{PBS}$ (Lonza). Cell membranes were spun down by centrifuging the solution at $18,000 \times g$ for 6 mins, and the supernatant was discarded. After repeating this process 4 times, the membrane pellet was white and was sonicated using a probe sonication (Fisher Sonic Dismembrator) at 20% amplitude for 6 s (pulse: 1 s on and 1 s off) on ice to generate RBCM vesicles. To quantify the concentration of RBCMs before sonication, the volume of RBCM pellet from 1 mL blood was measured. The collected RBCM pellet was lyophilized and weighed. The concentration of RBCMs before sonication was 20 mg/mL.

2.2. Preparation of RBCM scaffold

Alginate (PRONOVA SLM100) was dissolved in DI water at a concentration of 2 wt%. For 120 μL of 2 wt% alginate solution, 5 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Thermo Scientific) and 3 mg of N-hydroxysuccinimide (NHS) (ACROS Organics) were added and well mixed to activate carboxylic acid groups of

alginate. Of the activated alginate solution, 20 μL was mixed with 30 μL of RBCM vesicle solution (20 mg/mL). The mixture was transferred to a 1-mL syringe (BD, USA) and immediately put in a -20°C freezer for at least 12 h to form a disc-shaped scaffold. For mechanical property test, the amount of alginate was fixed at 20 μL (2 wt%), and RBCM vesicle solution was reduced from 30 μL to 20 μL and 15 μL to prepare RBCM scaffolds. PBS was added to make the total volume of 50 μL .

2.3. Preparation of methacrylate-alginate scaffolds

MA-alginate was synthesized as previously reported with a degree of substitution around 11% [15]. MA-alginate scaffolds were prepared by radical polymerization under subzero temperature. For 50 μL of 1 wt% MA-alginate water solution, 0.32 μL of tetramethylethylenediamine (TEMED, Fisher Scientific) and 1.25 μL of 10% ammonium persulfate (APS, Fisher Scientific) water solution were added. The solution was mixed and transferred to a 1-mL syringe and immediately put in a -20°C freezer for at least 12 h to form a disc-shaped scaffold.

2.4. Characterization of RBCM vesicles and RBCM scaffolds

The average size of RBCM vesicles was tested using a Zetasizer Nano ZS (Malvern Instruments, UK). The morphology of RBCM vesicles was imaged by transmission electron microscopy (TEM). Samples were prepared as follows: a carbon film-coated copper grid was cleaned by plasma, and 10 μL of vesicle solution was dropped on the grid. After 20 min, the grid was rinsed with 10 μL of DI water 3 times. Then 5 μL of 2% uranyl acetate water solution was dropped on the grid for 10 s before the stain solution was removed by a filter paper. The grid was then dried and imaged using a JEOL JEM2100 TEM at 200 kV.

The crosslinking of RBCM vesicles and alginate was characterized by Fourier transform infrared (FT-IR). FT-IR spectra of three samples including alginate (sample 1), the simple mixture of alginate and RBCM vesicles (sample 2), and RBCM scaffold (sample 3) were recorded by FT-IR spectrometer (Bruker, German) in the frequency range of $4000\text{--}400\text{ cm}^{-1}$ via the attenuated total reflection method. All three samples had the same amount of alginate, and sample 2 and 3 had the same amount of RBCM vesicles.

The macroporous structures of scaffolds were characterized by scanning electron microscopy (SEM) and confocal microscopy. For SEM sample preparation, scaffolds were flash-frozen using liquid nitrogen, cut with a scalpel, and lyophilized. Dry samples were mounted on stubs and imaged on an SEM (Zeiss Supra 50VP). For confocal microscopy, 1, 2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (RhB-DMPE) (Avanti, Alabama) was used to label RBCM vesicles, and 20 μg of fluorescein isothiocyanate labeled bovine serum albumin (FITC-BSA) (Sigma) was added during scaffold preparation to label the scaffold frame. RhB-DMPE-labeled RBCM vesicles were prepared as followed: 4 μL of RhB-DMPE in dimethyl sulfoxide (DMSO, Fisher Scientific) (2.5 mM) was added to 3.5 mL of PBS and the solution was then mixed with 0.5 mL of RBCM solution in PBS (RBCM from 1 mL blood) and incubated for 20 mins at room temperature for the lipid to insert into RBCM. RBCM was spun down and washed twice with PBS, and the pellet was sonicated to generate RBCM vesicles. Scaffolds

were washed in DI water and imaged using a laser scanning confocal microscope (Olympus FluoView FV1000).

Mechanical properties of scaffolds were characterized by atomic force microscopy (AFM). AFM-nanoindentation was performed using microspherical colloidal tips ($R \approx 100 \mu\text{m}$, nominal $k \approx 0.03 \text{ N/m}$, Arrow-TL1-SPL/Tipless/Silicon, NanoWorld, Neuchâtel, Switzerland) and a Dimension Icon AFM (BrukerNano, Santa Barbara, CA) at $10 \mu\text{m/s}$ indentation rate up to a maximum load of $\approx 100 \text{ nN}$. For each sample, nanoindentation was performed at least 12 locations to account for spatial heterogeneity. At each location, indentation was repeated three times, which confirmed the repeatability and the absence of permanent sample deformation. All indentation measurements were performed with each sample fully immersed in $1 \times \text{PBS}$. The effective indentation modulus, E_{ind} was calculated by fitting the loading portion of each F-D curve to the finite thickness corrected Hertz Model [38], in which Poisson's ratio, ν , was taken as 0.49 for highly swollen hydrogels [39].

2.5. Characterization of swelling ratio and water content of scaffolds

RBCM and MA-alginate scaffolds were immersed in PBS at 37°C under shaking at $100 \times \text{rpm}$. At designated time points, scaffolds were removed from PBS, excess PBS on scaffold surface was removed via pipetting, and scaffolds were weighed. Scaffolds were then rinsed in water, lyophilized, and weighed. The swelling ratio (Q_m) was calculated using the following equation:

$$Q_m = \frac{w_{wet} - w_{dry}}{w_{dry}}, \quad (1)$$

and water content was calculated using the following equation:

$$\text{Water content}(\text{wt}\%) = \frac{w_{wet} - w_{dry}}{w_{wet}}, \quad (2)$$

where w_{wet} is the swollen scaffold weight, and w_{dry} is the dry scaffold weight [40, 41]. The salt weight in PBS was subtracted for the swelling ratio and water content calculation.

2.6. Characterization of pyrene distribution in scaffolds and its release from scaffolds

Two μL of pyrene (Sigma) in DMSO at a concentration of 1 mg/mL was added to $50 \mu\text{L}$ of PBS or RBCM pellet solution (20 mg/mL in PBS). After sonication to generate RBCM vesicles, emission spectra of pyrene in PBS and RBCM vesicle solution were obtained using a plate reader (Biotek) with an excitation at 320 nm . MA-alginate scaffolds encapsulating $2 \mu\text{g}$ of pyrene were prepared by adding $2 \mu\text{L}$ of pyrene DMSO solution (1 mg/mL) to $50 \mu\text{L}$ of MA-alginate ($1 \text{ wt}\%$) before cryogelation. For the preparation of RBCM scaffolds encapsulating $2 \mu\text{g}$ of pyrene, $8 \mu\text{L}$ of pyrene DMSO solution (1 mg/mL) was added to $120 \mu\text{L}$ RBCM solution (20 mg/mL in PBS). The solution was sonicated to generate RBCM vesicles, and $30 \mu\text{L}$ of this RBCM vesicle solution was mixed with $20 \mu\text{L}$ of EDC and NHS-

activated alginate (2 wt% in water), which was transferred to a 1-mL syringe and put in a -20°C freezer for cryogelation. Emission spectra of pyrene in those scaffolds were recorded with an excitation at 320 nm. The emission spectra were normalized by setting the peak value as 1.

For the release study, RBCM scaffolds encapsulating 2 μg of pyrene were washed in PBS (5 mL for each scaffold) for 1 h. Afterwards, each scaffold was transferred to a 2-mL tube with 1 mL of PBS with 10 % fetal bovine serum (FBS, ATCC) and 1 % penicillin-streptomycin (PS, ATCC) at 37°C under shaking at $100 \times \text{rpm}$. At designated time points, the buffer was removed from each tube, and fresh buffer was added. Fluorescence intensity at 390 nm with an excitation at 320 nm was used to quantify the concentration of pyrene released in buffer solutions.

2.7. In vitro culture of bone marrow-derived macrophages (BMDMs)

BMDMs were cultured according to reported methods [42, 43]. In brief, female C57BL/6J mice (aged 8–10 weeks) were sacrificed, and femurs and tibias were collected. A syringe with a 29 G needle filled with Dulbecco's Modified Eagle Medium (DMEM, Lonza) was used to flush out the bone marrow. RBC lysis buffer (BioLegend) was used to remove RBCs. Cells were cultured in DMEM supplemented with 10% FBS, 1% PS, 50 μM of β -mercaptoethanol, and 20 ng/mL of M-CSF in petri dish (BD) for 7 days. Half of the media was replaced with fresh media on day 2 and day 5. On day 7, some cells were treated with LPS (100 ng/mL, Sigma) and interferon- γ (20 ng/mL, Peprotech) for 24 h for M1 polarization, while some were treated with interleukin-4 (20 ng/mL, Peprotech) for 24 h to differentiate into M2 macrophages. Cells were detached with 3 mM EDTA in PBS solution and used for future steps.

2.8. In vivo cell infiltration study

Female C57BL/6J mice aged 8–10 weeks were used for *in vivo* studies. Frozen MA-alginate and RBCM scaffolds were thawed and washed in DI water for 30 min and in saline for another 30 min. The mice were randomly selected into two groups. Two of the same type of scaffolds were subcutaneously (s.c.) injected into each mouse with one on the left side and one on the right side under anesthesia. After 1, 4, and 10 days, mice from each group were sacrificed, and the scaffolds were collected. The scaffolds were digested in 1 mL of 250 U/mL collagenase II (Worthington, NJ) solution for 30 min at 37°C . Afterwards, scaffolds were disrupted using pipetting and were drained through a 70 μm nylon cell strainer (VWR). Cells were then spun down at $1,500 \times \text{rpm}$ for 5 min, washed with PBS, and counted using a hemocytometer. For each mouse, cells isolated from two scaffolds were combined, and 0.25 million of those cells were used for staining and flow cytometry analysis. For staining, scaffold cells and BMDMs were incubated with anti-CD16/32 (BioLegend) for 15 min at 4°C to block Fc receptors. Then cells were stained with several antibodies including APC-conjugated CD11c (BioLegend), FITC-conjugated F4/80 (BioLegend), PE-conjugated Ly6G (BioLegend), PE/Cy7-conjugated PD-L1 (BioLegend), PerCP/Cy5.5-conjugated MHC-II (BioLegend), and APC/Cy7-conjugated CD86 (BioLegend) for 30 min at 4°C . After staining, cells were washed twice with PBS containing 4% FBS and characterized on a flow cytometer (BD Bioscience). FlowJo (Tree Star) was used to analyze the data. For measuring

the concentrations of cytokines in scaffolds, scaffolds were collected on day 4 post scaffold injection, and proteins in scaffolds were extracted using Tissue Extraction Reagent (Invitrogen) following manufacturer instruction. Meanwhile, proteins from spleens, livers, inguinal and axillary lymph nodes (LNs) were also extracted and used as controls. Cytokine concentrations were measured by using IL-10, IL-12, and TNF- α ELISA kits (Peprotech). For histology analysis, mice from each group were sacrificed after 2 weeks and 4 weeks. Scaffolds and surrounding skin tissues were retrieved and embedded in paraffin. Samples were sectioned and stained for hematoxylin-eosin (H&E) stain at the Histotechnology facility of the Wistar Institute. The images were taken by using a Leica DM4000B Microscope.

2.9. Real-time polymerase chain reaction (qPCR) analysis of cytokine expression

Freshly isolated cells from scaffolds and BMDMs were stored in TRIzol (Life Technologies), and chloroform (Fisher Scientific) was used to extract RNA from cells. TURBO DNA-free Kit (Invitrogen) was used to remove any trace amounts of DNA. The concentration of RNA was determined by a NanoDrop 1000 Spectrophotometer (Thermo Scientific). High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to prepare cDNA. PowerUp SYBR Green Master Mix (Applied Biosystems) was used to carry out qPCR on QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). Primer sequences were as follows: arginase-1 (Arg-1) (F: TTGGGTGGATGCTCACACTG; R: GTACACGATGTCTTTGGCAGA) and inducible nitric oxide synthase (iNOS) (F: ACATCGACCCGTCCACAGTAT; R: CAGAGGGGTAGGCTTGTCTC). All data were presented as relative to M0 BMDMs.

2.10. In vivo study of cell membrane vesicle uptake by infiltrated macrophages in scaffolds and scaffold degradation.

Isolated RBCs were washed three times with PBS and resuspended in PBS (0.15 mL blood per 1 mL PBS). Alexa Fluor 647 NHS ester (Life Technologies) was dissolved in DMSO (ATCC) at a concentration of 20 mg/mL. The dye solution was then added to the RBC solution (10 μ M), and the well-mixed solution was incubated at room temperature for 30 mins. RBCs were washed twice with PBS, and the conjugation of Alexa Fluor 647 on RBCs was characterized by confocal microscopy. Alexa Fluor 647-labeled RBCM scaffolds were prepared following the same method described in section 2.2. To study the uptake of RBCM vesicles in RBCM scaffolds by infiltrated macrophages, mice were subcutaneously injected with either 2 RBCM scaffolds (control) or 2 Alexa Fluor 647-labeled RBCM scaffolds. After 2 days, 10 days, and 1 month, scaffolds were collected and digested in 1 mL of 250 U/mL collagenase II solution for 30 min at 37 °C. The fluorescence intensity of scaffold solutions at 655 nm was recorded with an excitation at 625 nm. Cells were isolated and stained with two groups of antibodies: the first group included FITC-conjugated F4/80, PE-conjugated Ly6G, PE/Cy7-conjugated PD-L1, PerCP/Cy5.5-conjugated MHC-II, and APC/Cy7-conjugated CD86, and the second group replaced PE-conjugated Ly6G with PE-conjugated CD11c (BioLegend). Characterization and analysis were done as described in section 2.8.

2.11. Statistical analysis

Values are presented as mean $\pm$ SD. Student's t-test (two-sample assuming equal variances) was used to analyze statistical significance between two groups, and one-way ANOVA with Tukey's post hoc multiple comparison test was used to analyze statistical significance among three or more groups. A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Fabrication and characterization of RBCM scaffolds

To fabricate cell-derived porous scaffolds, our governing strategy was to chemically crosslink cell components, particularly cell membrane vesicles via a cryogelation method [24]. Ultrapure alginate was utilized as the linker of vesicles. The reaction between carboxyl groups on alginate and primary amines on cell membrane vesicles was selected for crosslinking to minimize the effect of crosslinking reaction on scaffold biocompatibility. During crosslinking, EDC and NHS-activated carboxylic groups on alginate either react with amine groups on RBCM vesicles to crosslink vesicles or revert to carboxylic acids via hydrolysis without generating any non-natural functional groups (Fig. 1). When vesicles are crosslinked at -20°C , macroporous structures form due to water crystallization. To prepare RBCM scaffolds, mouse RBCMs were isolated, and RBCM vesicles were generated by probe sonication of RBCMs. The vesicles had a size of around 170 nm with a narrow distribution (PDI ~ 0.15) as measured via dynamic light scattering (DLS) (Fig. 2A). The TEM image shows a spherical morphology of RBCM vesicles with a size matching the result from DLS (Fig. 2B). The structures inside the vesicles are likely debris generated during TEM sample preparation [44]. The RBCM vesicles (0.6 mg) were then crosslinked with activated alginate (0.4 mg) to form a macroporous cryogel. The solutions did not form a cryogel when either alginate or RBCM vesicles were missing. Fig. 2C shows a disc-shaped RBCM scaffold with RhB-DMPE-labeled RBCM vesicles. The crosslinking was confirmed by FT-IR as shown in Fig. S1. For alginate, peaks at 1609 cm^{-1} and 1403 cm^{-1} are asymmetric and symmetric stretching peaks of carboxylic acid groups. The peak at 1403 cm^{-1} does not change in the samples with only a mixture of inactivated alginate and RBCM vesicles, while the peak at 1609 cm^{-1} shifts to a higher wavelength due to the overlap with the amide I peak at 1648 cm^{-1} . However, the peak of RBCM scaffolds at 1403 cm^{-1} is reduced and the peaks of amide I at 1648 cm^{-1} and amide II at 1539 cm^{-1} become more obvious and sharper, due to the consumption of carboxylic acid groups and formation of amide bonds during the crosslinking of alginate and RBCM vesicles. The pore size of RBCM scaffolds was around $70\text{ }\mu\text{m}$ in an SEM image of a cross-sectioned scaffold (Fig. 2D). To further characterize the structure of RBCM scaffolds, RBCM vesicles were labeled with RhB-DMPE, and FITC-BSA was added during the fabrication step to directly label the interconnected scaffold frame. The confocal microscopy images of the scaffold show a pore size similar to that observed in the SEM image, and the frame of the porous scaffold is composed of closely packed RBCM vesicles (Fig. 2E).

Next, the mechanical properties of RBCM scaffolds were measured via AFM-based nanoindentation. Macroporous MA-alginate scaffolds were employed as a control as they have been reported to possess a good biocompatibility and mechanical properties [15, 24].

The MA-alginate scaffolds were also prepared through cryogelation, but via radical polymerization of MA-alginate. RBCM scaffolds had a lower effective indentation modulus, E_{ind} (1.81 ± 0.34 kPa) than MA-alginate scaffolds (5.32 ± 0.82 kPa) (Fig. 2F). The lower modulus may result from a lower crosslinking density and deformable RBCM vesicles. The crosslinking density in RBCM scaffolds is likely lower than that in alginate scaffolds because of inefficient access of alginate to amine groups on cell membranes. On the other hand, RBCM scaffolds are composed of crosslinked membrane vesicles, which are highly deformable under forces [45].

We further examined how the amount of RBCM vesicles affected the scaffold mechanical properties. Scaffolds were prepared with various amounts of RBCM vesicles and a fixed amount of alginate. The scaffold modulus increased significantly with the ratio of RBCM vesicles/alginate from 1.05 kPa to 1.81 kPa when the amount of vesicles doubled (Fig. 2G), likely because of an increase in crosslinking density within the studied concentration range. The RBCM vesicles/alginate ratio of 1.5, which is the highest ratio that can be conveniently obtained, was used for the rest of the studies. One advantage of the MA-alginate scaffold is its shape-memory capability after injection [24]. It was found that RBCM scaffolds at 50 μ L could maintain their shape and internal structures after injection through a 16 gauge needle, providing a convenient administration method for *in vivo* evaluation and future applications (Fig. S2).

The swelling ratio of both scaffolds in PBS was studied at 37 °C under shaking at $100 \times$ rpm for 3 days. The swelling ratio of both scaffolds did not change significantly from 1 day to 3 days, and the swelling ratio of MA-alginate scaffolds ($74.8 \pm 3.1\%$) at day 3 was larger than that of RBCM scaffolds ($45.2 \pm 4.2\%$) due to the lower dry weight of MA-alginate scaffolds (Fig. S3A). Both scaffolds showed high water content, up to $\sim 98\%$, which is in accordance with their macroporous structures (Fig. S3B).

3.2. Encapsulation and controlled release of hydrophobic model drug pyrene

RBCMs consist of lipid bilayers, which have a hydrophobic region of fatty acid chains, allowing the encapsulation of hydrophobic drugs within the bilayers. A hydrophobic fluorescent molecule, pyrene, was used as a model drug to study its interaction with RBCM vesicles in solutions and in scaffolds. The emission wavelength of pyrene monomers peaks in the range of 370 nm to 400 nm, while that of pyrene excimers peaks in the range of 440 nm to 500 nm [46]. This distinction in the emission spectra of pyrene enables us to conveniently probe its molecular aggregation states. In PBS, pyrene showed a wide emission peak around 456 nm (Fig. 3A), which corresponds to the emission spectrum of excimers. In contrast, pyrene in RBCM vesicle solutions exhibited a narrow emission peak at 390 nm and low emission intensity at 456 nm (Fig. 3A), similar to the reported emission spectrum of pyrene monomers [47–49]. The ratio of pyrene monomers and excimers can be quantified by the emission intensity ratio at 390 nm and 456 nm of a pyrene spectrum [50–52]. I_{390}/I_{456} of pyrene in RBCM vesicle solutions had a value of 5.61 ± 0.05 , while it was 0.27 ± 0.01 in PBS (Fig. 3B). This demonstrates that RBCM vesicles dispersed pyrene homogeneously in solution, and pyrene clustered together in PBS due to their hydrophobicity. Next we studied whether RBCM vesicles could still disperse pyrene molecules in RBCM scaffolds. While

pyrene in MA-alginate scaffolds showed a comparable emission pattern to that in PBS, the emission spectrum in RBCM scaffolds was similar to that in RBCM vesicle solutions (Fig. 3C). The values of I_{390}/I_{456} were 6.51 ± 0.30 and 0.27 ± 0.08 in RBCM scaffolds and MA-alginate scaffolds, respectively (Fig. 3D), demonstrating that pyrene was well-dispersed in RBCM scaffolds.

The encapsulation and release of pyrene in RBCM scaffolds were then evaluated. Under the investigated concentration, the encapsulation efficiency of pyrene in RBCM scaffolds was $99.48 \pm 0.14\%$. The release of pyrene from the scaffolds in PBS supplemented with 10% FBS showed a fast release of $17.41 \pm 2.32\%$ in the first 8 h and then a steady release of $30.46 \pm 0.99\%$ over the rest of the week (Fig. 3E). This demonstrates that RBCM scaffolds are promising for sustained release of hydrophobic drugs.

3.3. Identification of immune cells inside scaffolds in vivo

The types of immune cells that come in contact with scaffolds are critical to the immune responses that the scaffolds elicit *in vivo*. Neutrophils and M1 macrophages are associated with inflammation, while M2 macrophages possess anti-inflammatory functions in wound healing and tissue regeneration [53]. The identification of cell phenotypes within scaffolds can provide direct information on scaffold biocompatibility and their potential in tissue regeneration and immune modulation [54–56]. We have characterized the recruited cells in subcutaneously injected RBCM scaffolds in mice and compared the results to MA-alginate scaffolds. One day after injection, similar numbers of cells migrated to both scaffolds with 0.57 ± 0.10 million cells per RBCM scaffold and 0.53 ± 0.21 million cells per MA-alginate scaffold (Fig. 4A). The cell number in RBCM scaffolds increased to 0.89 ± 0.11 million on day 4 and remained the same from day 4 to day 10 (0.87 ± 0.16 million), while that in MA-alginate scaffolds slightly decreased to 0.37 ± 0.20 million on day 4 and to 0.36 ± 0.06 million on day 10. The cell numbers in RBCM scaffolds were significantly higher than those in MA-alginate scaffolds on day 4 and day 10. We then identified cell types via surface marker staining and flow cytometry analysis. Dendritic cells (DCs) ($CD11c^+F4/80^-$), macrophages ($F4/80^+$), and neutrophils ($Ly6G^+$) in scaffolds were analyzed. The gating strategy for each cell population is shown in Fig. S4. In both scaffolds, the percentages of DCs decreased with time 1 day after s.c. injection of the scaffolds (Fig. 4B). The percentages of DCs in RBCM scaffolds were significantly lower than those in MA-alginate scaffolds on day 4 and day 10. The numbers of DCs in both scaffolds also showed a decreasing trend with time, with 6×10^4 DCs on day 1 and around 2×10^4 DCs on day 10 in RBCM scaffolds. The percentages and numbers of neutrophils in both scaffolds exhibited similar patterns to DCs, except that there was no significant difference between the two scaffolds. The low number of infiltrated neutrophils indicates that RBCM scaffolds, similar to MA-alginate scaffolds, cause minimal acute inflammatory responses in mice [15]. Macrophages were the most abundant cells in both scaffolds throughout the study period. The percentage of macrophages in RBCM scaffolds gradually increased and reached $73.3 \pm 1.2\%$ in RBCM scaffolds on day 10, significantly higher than $63.1 \pm 2.1\%$ in MA-alginate scaffolds (Fig. 4B and C). The macrophage numbers were around 0.25 million in both scaffolds on day 1. They increased to 0.60 ± 0.03 million on day 4 and 0.64 ± 0.11 million on day 10 in RBCM scaffolds, significantly higher than the macrophage population

that remained the same in MA-alginate scaffolds. Overall, both scaffolds exhibited a good biocompatibility as shown by the low number of infiltrated neutrophils. In terms of cell populations, the major difference between these two scaffolds was the significantly increased macrophages in RBCM scaffolds after day 1.

3.4. Investigation of the phenotype of infiltrated macrophages

Many monocytes that migrate from the blood into tissues differentiate into macrophages. Monocytes become proinflammatory M1 macrophages under classical activation. These cells are associated with immunostimulation, pathogen and debris clearance, and early stage tissue regeneration. Monocytes can also differentiate into M2 macrophages through the alternative activation, becoming immune suppressive [53, 57]. To identify the subtype of macrophages in the scaffolds, we cultured M0, M1, and M2 BMDMs and used them as controls. It is worth noting that the *in vitro* differentiated cells are not fully analogous to their counterparts *in vivo* because cells *in vitro* and *in vivo* are stimulated in different environments [58]. Compared with M0 and M1 BMDMs, cells from both scaffolds at all three time points starting from day 1 showed a high expression of an M2 gene marker Arg-1, similar to those observed in M2 BMDMs, while the expression of an M1 gene marker, iNOS in the scaffold cells was more than 100 times lower than that in M1 BMDMs (Fig. 5A) [43, 59]. To further characterize the activation status of the scaffold macrophages, cell surface markers CD86, MHC-II, and PD-L1 were stained and analyzed with flow cytometry. CD86 and MHC-II are immune stimulatory markers of macrophages [60, 61], and the expression of PD-L1 is associated with immune suppression [62, 63]. Approximately 40% of M0 and M2 BMDMs and 100% of M1 BMDMs were CD86⁺ (Fig. S5). The percentages of CD86⁺ macrophages (F4/80⁺CD86⁺) in both scaffolds were much lower than that of M1 BMDMs and gradually decreased. By day 10, the percentages of CD86⁺ macrophages in both scaffolds were even significantly lower than those of M0 and M2 BMDMs. The mean fluorescence intensity (MFI) of CD86 among macrophages also showed a significant downregulation of CD86 over time. The expression of MHC-II on macrophages in both scaffolds was much lower than that of M1 BMDMs, and was similar to those of M0 and M2 BMDMs, except those in MA-alginate scaffolds on day 10 (Fig. 5B). Those cells expressed significantly higher levels of MHC-II than those in RBCM scaffolds. The reason behind this upregulation is still unclear as one would expect a gradual transition to M2 phenotype.

Although more than 90% of M0, M1, and M2 BMDMs were PD-L1⁺, M1 BMDMs expressed a much higher level of PD-L1 than M0 and M2 BMDMs (Fig. 5C), which is similar to a previous report [64]. Macrophages in both scaffolds did not show the pattern of PD-L1 expression in M1 BMDMs at any of the detection time points. Macrophages in RBCM scaffolds showed a significantly higher percentage of PD-L1⁺ cells than those in MA-alginate scaffolds after 4 days. The MFI of macrophage PD-L1 in RBCM scaffolds was significantly higher than that of MA-alginate scaffolds on day 4. Overall, the analysis of surface markers supports the result in the gene expression study that macrophages in both scaffolds had an M2-like phenotype from day 1, and indicates macrophages in RBCMs scaffolds were more anti-inflammatory than those in MA-alginate scaffolds.

3.5. Biocompatibility of scaffolds

The short-term biocompatibility of scaffolds was also characterized by measuring the cytokine concentrations within the scaffolds on day 4 and was then compared to cytokine concentrations in different organs including spleen, liver, and LNs. The concentrations of IL-10, an anti-inflammatory cytokine, in both RBCM and MA-alginate scaffolds were significantly higher than those in spleen, liver, and LNs (Fig. 6A). The concentrations of IL-12 and TNF- α , two pro-inflammatory cytokines, in both RBCM and MA-alginate scaffolds were comparable to those in spleen, liver, and LNs, and they were more than 10 times lower than IL-10 concentrations (Fig. 6A and Fig. S6). This indicates that both RBCM and MA-alginate scaffolds generated an anti-inflammatory environment on day 4 after their s.c. injections. The anti-inflammatory effects of RBCM scaffolds have not been achieved by other methods [65]. For example, as we previously showed, encapsulation and controlled release of anti-inflammatory drugs prevent immune cells from migrating into biomaterials scaffolds, whereas proper immune cells are important for tissue regeneration. We further studied biocompatibility of both scaffolds at 2 weeks and 1 month by H&E staining. Both scaffolds showed a thin fibrotic capsule with 2 or 3 cell layers compared to the bare skin control (Fig. 6B), demonstrating their good mid-term biocompatibility.

3.6 In vivo uptake of RBCM vesicles from RBCM scaffolds by infiltrated macrophages

The engulfment of apoptotic cell materials by macrophages induces their polarization toward anti-inflammatory phenotypes [66, 67]. RBCM vesicles contain phosphatidylserine, an apoptotic signaling molecule. Therefore, the demonstration of RBCM vesicle uptake by infiltrated macrophages may provide an explanation of the observed anti-inflammatory effects of RBCM scaffolds. We studied the uptake of RBCM vesicles by infiltrated macrophages at various time points. To label the vesicles, RBC membrane proteins were conjugated with Alexa Fluor 647. Fig. 7A shows that the conjugation of Alexa Fluor 647 did not affect the biconcave morphology of RBCs. RBCM scaffolds were then prepared using Alexa Fluor 647-labeled RBCs and injected into mice for the uptake study. The total number of cells infiltrated into scaffolds was around 0.85 million 2 and 10 days after scaffold injection, and decreased to 0.56 million after 1 month possibly due to the emigration of recruited cells (Fig. S7A). The majority of the cells were macrophages, and the number of neutrophils became minimal after 2 days (Fig. 7B and Fig. S7B). Therefore, we focused on the examination of RBCM vesicle uptake by macrophages.

Compared with macrophages from unlabeled RBCM scaffolds as a control, macrophages from Alexa Fluor 647-labeled scaffolds showed increased Alexa Fluor 647 signal (Fig. 7C). The shifts of the whole histogram curves indicate that most macrophages have internalized RBCM vesicles. Macrophages engulfed a large amount of RBCM vesicles from day 2 to day 10 and did not internalize more after 10 days (Fig. 7C and 7D). We also characterized the change of scaffold fluorescence intensity with time as an indicator of scaffold degradation *in vivo*. Compared with the intensity of scaffolds before injection, the intensity of scaffolds decreased by 4.6% after 2 days and by 34.2% after 10 days (Fig. S8). The intensity did not change significantly from 10 days to 1 month, the furthest timepoint in this study. The kinetics of vesicle uptake and scaffold degradation are consistent. Recruited macrophages

possibly engulfed RBCM vesicles exposed on the scaffold framework quickly and could not efficiently internalize those that are well-crosslinked.

4. Conclusion

In summary, we have developed a new class of biological materials: cell membrane-derived hydrogel scaffolds by using EDC and NHS-activated alginate to crosslink RBCM vesicles. After cryogelation, macroporous RBCM scaffolds were formed with closely packed RBCM vesicles as the scaffold frame. The soft scaffolds can maintain its internal structure after injection. Unlike most highly biocompatible scaffolds made of hydrophilic polymers, RBCM scaffolds can encapsulate hydrophobic molecules and have the potential to release hydrophobic drugs in a sustained manner. Similar to MA-alginate scaffolds, RBCM scaffolds present a good biocompatibility in the studied time frame. There were significantly higher numbers of macrophages in RBCM scaffolds than in MA-alginate scaffolds 4 days after s.c. injection of the scaffolds. Based on gene expression and surface markers, the macrophages in both scaffolds exhibited an M2-like phenotype. Macrophages from RBCM-alginate scaffolds showed a higher percentage of PD-L1⁺ cells after 4 days and lower MHC-II expression after 10 days compared with those from MA-alginate scaffolds, indicating the cells may have a stronger immune suppressive ability. Because RBCM scaffolds can recruit a higher number of anti-inflammatory macrophages, we expect this type of biological materials will find wide applications in tissue regeneration and tolerance induction. It remains to be determined which specific interaction(s) between RBCM vesicles and recruited cells caused the difference, even though our data indicates that it may be associated with the uptake of RBCM vesicles by macrophages. Since wound healing requires orchestrated sequential balance of M1 and M2 macrophages [59], our RBCM scaffolds may be particularly helpful for wounds with excessive inflammation. Other methods may not generate a similar anti-inflammatory effect. This study paves the way to explore scaffolds derived from other cells that present receptors and/or contain cytokines in regulating immune cells or cells critical for tissue regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a National Institutes of Health Grant R21AI133372, a seed grant from the Clinical & Translational Research Institute (CTRI) at Drexel University, funding from PA Department of Health, CURE grant program, and a JDRF grant 1-PNF-2018-658-A-N. The authors thank Dr. Kara Spiller for the helpful discussion about macrophages.

References

- [1]. Ott HC, Clippinger B, Conrad C, Schuetz C, Pomerantseva I, Ikonomidou L, Kotton D, Vacanti JP, Regeneration and orthotopic transplantation of a bioartificial lung. *Nat. Med* 16(8) (2010) 927–933. [PubMed: 20628374]
- [2]. Ott HC, Matthiesen TS, Goh SK, Black LD, Kren SM, Netoff TI, Taylor DA, Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat. Med* 14(2) (2008) 213–221. [PubMed: 18193059]

- [3]. Uygun BE, Soto-Gutierrez A, Yagi H, Izamis ML, Guzzardi MA, Shulman C, Milwid J, Kobayashi N, Tilles A, Berthiaume F, Hertl M, Nahmias Y, Yarmush ML, Uygun K, Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix, *Nat. Med* 16(7) (2010) 814–820. [PubMed: 20543851]
- [4]. Quint C, Kondo Y, Manson RJ, Lawson JH, Dardik A, Niklason LE, Decellularized tissue-engineered blood vessel as an arterial conduit, *Proc. Natl. Acad. Sci. U. S. A* 108(22) (2011) 9214–9219. [PubMed: 21571635]
- [5]. Vega SL, Kwon MY, Song KH, Wang C, Mauck RL, Han L, Burdick JA, Combinatorial hydrogels with biochemical gradients for screening 3D cellular microenvironments, *Nat. Commun* 9 (2018).
- [6]. Isa ILM, Abbah SA, Kilcoyne M, Sakai D, Dockery P, Finn DP, Pandit A, Implantation of hyaluronic acid hydrogel prevents the pain phenotype in a rat model of intervertebral disc injury, *Sci. Adv* 4(4) (2018).
- [7]. Khetan S, Guvendiren M, Legant WR, Cohen DM, Chen CS, Burdick JA, Degradation-mediated cellular traction directs stem cell fate in covalently crosslinked three-dimensional hydrogels, *Nat. Mater* 12(5) (2013) 458–465. [PubMed: 23524375]
- [8]. Silva JP, Goncalves C, Costa C, Sousa J, Silva-Gomes R, Castro AG, Pedrosa J, Appelberg R, Gama FM, Delivery of LLKKK18 loaded into self-assembling hyaluronic acid nanogel for tuberculosis treatment, *J. Controlled Release* 235 (2016) 112–124.
- [9]. Khatun Z, Nurunnabi M, Nafiujjaman M, Reeck GR, Khan HA, Cho KJ, Lee YK, A hyaluronic acid nanogel for photo-chemo theranostics of lung cancer with simultaneous light-responsive controlled release of doxorubicin, *Nanoscale* 7(24) (2015) 10680–10689. [PubMed: 26030737]
- [10]. Hong F, Zhang F, Liu Y, Yan H, DNA Origami: Scaffolds for Creating Higher Order Structures, *Chem. Rev* 117(20) (2017) 12584–12640. [PubMed: 28605177]
- [11]. Praetorius F, Kick B, Behler KL, Honemann MN, Weuster-Botz D, Dietz H, Biotechnological mass production of DNA origami, *Nature* 552(7683) (2017) 84–87. [PubMed: 29219963]
- [12]. Chen HR, Li RX, Li SM, Andreasson J, Choi JH, Conformational Effects of UV Light on DNA Origami, *J. Am. Chem. Soc* 139(4) (2017) 1380–1383. [PubMed: 28094518]
- [13]. Hu CMJ, Zhang L, Aryal S, Cheung C, Fang RH, Zhang LF, Erythrocyte membrane-camouflaged polymeric nanoparticles as a biomimetic delivery platform, *Proc. Natl. Acad. Sci. U. S. A* 108(27) (2011) 10980–10985. [PubMed: 21690347]
- [14]. A Trends Guide Zhou H, Fan ZY, Lemons PK, Cheng H, A Facile Approach to Functionalize Cell Membrane-Coated Nanoparticles, *Theranostics* 6(7) (2016) 1012–1022. [PubMed: 27217834]
- [15]. Fan Z, Li PY, Deng J, Bady SC, Cheng H, Cell membrane coating for reducing nanoparticle-induced inflammatory responses to scaffold constructs, *Nano Res* (2018).
- [16]. Hu CMJ, Fang RH, Luk BT, Zhang LF, Nanoparticle-detained toxins for safe and effective vaccination, *Nat. Nanotechnol* 8(12) (2013) 933–938. [PubMed: 24292514]
- [17]. Hu CMJ, Fang RH, Wang KC, Luk BT, Thamphiwatana S, Dehaini D, Nguyen P, Angsantikul P, Wen CH, Kroll AV, Carpenter C, Ramesh M, Qu V, Patel SH, Zhu J, Shi W, Hofman FM, Chen TC, Gao WW, Zhang K, Chien S, Zhang LF, Nanoparticle biointerfacing by platelet membrane cloaking, *Nature* 526(7571) (2015) 118–121. [PubMed: 26374997]
- [18]. Chen Z, Zhao PF, Luo ZY, Zheng MB, Tian H, Gong P, Gao GH, Pan H, Liu LL, Ma AQ, Cui HD, Ma YF, Cai LT, Cancer Cell Membrane-Biomimetic Nanoparticles for Homologous-Targeting Dual-Modal Imaging and Photothermal Therapy, *ACS Nano* 10(11) (2016) 10049–10057. [PubMed: 27934074]
- [19]. Kang T, Zhu QQ, Wei D, Feng JX, Yao JH, Jiang TZ, Song QX, Wei XB, Chen HZ, Gao XL, Chen J, Nanoparticles Coated with Neutrophil Membranes Can Effectively Treat Cancer Metastasis, *ACS Nano* 11(2) (2017) 1397–1411. [PubMed: 28075552]
- [20]. Yang R, Xu J, Xu LG, Sun XQ, Chen Q, Zhao YH, Peng R, Liu Z, Cancer Cell Membrane-Coated Adjuvant Nanoparticles with Mannose Modification for Effective Anticancer Vaccination, *ACS Nano* 12(6) (2018) 5121–5129.
- [21]. Hu QY, Sun WJ, Qian CG, Wang C, Bombardieri HN, Gu Z, Anticancer Platelet-Mimicking Nanovehicles, *Adv. Mater* 27(44) (2015) 7043–7050. [PubMed: 26416431]

- [22]. Zhang XD, Wang C, Wang JQ, Hu QY, Langworthy B, Ye YQ, Sun WJ, Lin J, Wang TF, Fine J, Cheng H, Dotti G, Huang P, Gu Z, PD-1 Blockade Cellular Vesicles for Cancer Immunotherapy, *Adv. Mater* 30(22) (2018).
- [23]. Ali OA, Huebsch N, Cao L, Dranoff G, Mooney DJ, Infection-mimicking materials to program dendritic cells in situ, *Nat. Mater* 8(2) (2009) 151–158. [PubMed: 19136947]
- [24]. Bencherif SA, Sands RW, Bhatta D, Arany P, Verbeke CS, Edwards DA, Mooney DJ, Injectable preformed scaffolds with shape-memory properties, *Proc. Natl. Acad. Sci. U. S. A* 109(48) (2012) 19590–19595. [PubMed: 23150549]
- [25]. Griffin DR, Weaver WM, Scumpia PO, Di Carlo D, Segura T, Accelerated wound healing by injectable microporous gel scaffolds assembled from annealed building blocks, *Nat. Mater* 14(7) (2015) 737–744. [PubMed: 26030305]
- [26]. Liang YK, Kiick KL, Liposome-Cross-Linked Hybrid Hydrogels for Glutathione-Triggered Delivery of Multiple Cargo Molecules, *Biomacromolecules* 17(2) (2016) 601–614. [PubMed: 26751084]
- [27]. Fuhrmann G, Chandrawati R, Parmar PA, Keane TJ, Maynard SA, Bertazzo S, Stevens MM, Engineering Extracellular Vesicles with the Tools of Enzyme Prodrug Therapy, *Adv. Mater* 30(15) (2018).
- [28]. Peppas NA, Hilt JZ, Khademhosseini A, Langer R, Hydrogels in biology and medicine: From molecular principles to bionanotechnology, *Adv. Mater* 18(11) (2006) 1345–1360.
- [29]. Zhang YS, Arneri A, Bersini S, Shin SR, Zhu K, Goli-Malekabadi Z, Aleman J, Colosi C, Busignani F, Dell'Erba V, Bishop C, Shupe T, Demarchi D, Moretti M, Rasponi M, Dokmeci MR, Atala A, Khademhosseini A, Bioprinting 3D microfibrinous scaffolds for engineering endothelialized myocardium and heart-on-a-chip, *Biomaterials* 110 (2016) 45–59. [PubMed: 27710832]
- [30]. Purcell BP, Lobb D, Charati MB, Dorsey SM, Wade RJ, Zellars KN, Doviak H, Pettaway S, Logdon CB, Shuman JA, Freels PD, Gorman JH, Gorman RC, Spinale FG, Burdick JA, Injectable and bioresponsive hydrogels for on-demand matrix metalloproteinase inhibition, *Nat. Mater* 13(6) (2014) 653–661. [PubMed: 24681647]
- [31]. Oh BHL, Bismarck A, Chan-Park MB, Injectable, Interconnected, High-Porosity Macroporous Biocompatible Gelatin Scaffolds Made by Surfactant-Free Emulsion Templating, *Macromol. Rapid Commun* 36(4) (2015) 364–372. [PubMed: 25504548]
- [32]. Akilbekova D, Shaimerdenova M, Adilov S, Berillo D, Biocompatible scaffolds based on natural polymers for regenerative medicine, *Int. J. Biol. Macromol* 114 (2018) 324–333. [PubMed: 29578021]
- [33]. Zhang L, Cao Z, Bai T, Carr L, Ella-Menye J-R, Irvin C, Ratner BD, Jiang S, Zwitterionic hydrogels implanted in mice resist the foreign-body reaction, *Nat. Biotechnol* 31 (2013) 553–556. [PubMed: 23666011]
- [34]. Li PY, Fan ZY, Cheng H, Cell Membrane Bioconjugation and Membrane-Derived Nanomaterials for Immunotherapy, *Bioconjug. Chem* 29(3) (2018) 624–634. [PubMed: 29323870]
- [35]. Wang Q, Cheng H, Peng HS, Zhou H, Li PY, Langer R, Non-genetic engineering of cells for drug delivery and cell-based therapy, *Adv. Drug Del. Rev* 91 (2015) 125–140.
- [36]. Stephan MT, Irvine DJ, Enhancing cell therapies from the outside in: Cell surface engineering using synthetic nanomaterials, *Nano Today* 6(3) (2011) 309–325. [PubMed: 21826117]
- [37]. Cheng H, Byrsk-Bishop M, Zhang CT, Kastrup CJ, Hwang NS, Tai AK, Lee WW, Xu XY, Nahrendorf M, Langer R, Anderson DG, Stem cell membrane engineering for cell rolling using peptide conjugation and tuning of cell-selectin interaction kinetics, *Biomaterials* 33(20) (2012) 5004–5012. [PubMed: 22494889]
- [38]. Dimitriadis EK, Horkay F, Maresca J, Kachar B, Chadwick RS, Determination of elastic moduli of thin layers of soft material using the atomic force microscope, *Biophys. J* 82(5) (2002) 2798–2810. [PubMed: 11964265]
- [39]. Ahearne M, Yang Y, El Haj AJ, Then KY, Liu K-K, Characterizing the viscoelastic properties of thin hydrogel-based constructs for tissue engineering applications, *J R Soc Interface* 2(5) (2005) 455–463. [PubMed: 16849205]

- [40]. Park H, Guo X, Temenoff JS, Tabata Y, Caplan AI, Kasper FK, Mikos AG, Effect of Swelling Ratio of Injectable Hydrogel Composites on Chondrogenic Differentiation of Encapsulated Rabbit Marrow Mesenchymal Stem Cells In Vitro, *Biomacromolecules* 10(3) (2009) 541–546. [PubMed: 19173557]
- [41]. Huglin MB, Yip DCF, An alternative method of determining the water content of hydrogels, *Makromol Chem. Rapid Commun* 8(5) (1987) 237–242.
- [42]. Makita N, Hizukuri Y, Yamashiro K, Murakawa M, Hayashi Y, IL-10 enhances the phenotype of M2 macrophages induced by IL-4 and confers the ability to increase eosinophil migration, *Int. Immunol* 27(3) (2015) 131–141. [PubMed: 25267883]
- [43]. McWhorter FY, Wang TT, Nguyen P, Chung T, Liu WF, Modulation of macrophage phenotype by cell shape, *Proc. Natl. Acad. Sci. U. S. A* 110(43) (2013) 17253–17258. [PubMed: 24101477]
- [44]. Fan Z, Zhou H, Li PY, Speer JE, Cheng H, Structural elucidation of cell membrane-derived nanoparticles using molecular probes, *J. Mater. Chem. B* 2(46) (2014) 8231–8238.
- [45]. Navot Y, Elastic membranes in viscous shear flow, *Phys. Fluids* 10(8) (1998) 1819–1833.
- [46]. Wu YL, Wang J, Zeng F, Huang SL, Huang J, Xie HT, Yu CM, Wu SZ, Pyrene Derivative Emitting Red or near-Infrared Light with Monomer/Excimer Conversion and Its Application to Ratiometric Detection of Hypochlorite, *ACS Appl. Mater. Interfaces* 8(2) (2016) 1511–1519. [PubMed: 26701212]
- [47]. Wilson JN, Kool ET, Fluorescent DNA base replacements: reporters and sensors for biological systems, *Org. Biomol. Chem* 4(23) (2006) 4265–4274. [PubMed: 17102869]
- [48]. Mahara A, Iwase R, Sakamoto T, Yamana K, Yamaoka T, Murakami A, Bispirene-conjugated 2'-O-methyloligonucleotide as a highly specific RNA-recognition probe, *Angew. Chem. Int. Ed. Engl* 41(19) (2002) 3648–3650. [PubMed: 12370918]
- [49]. Fan ZY, Wu JS, Liu WM, Ma JJ, Sun JY, Wang PF, Thiol-selective sensor based on intramolecular energy transfer between a bichromophoric system, *Tetrahedron* 69(23) (2013) 4536–4540.
- [50]. Xu Z, Singh NJ, Lim J, Pan J, Kim HN, Park S, Kim KS, Yoon J, Unique Sandwich Stacking of Pyrene-Adenine-Pyrene for Selective and Ratiometric Fluorescent Sensing of ATP at Physiological pH, *J. Am. Chem. Soc* 131(42) (2009) 15528–15533. [PubMed: 19919166]
- [51]. Focsaneanu KS, Scaiano JC, Potential analytical applications of differential fluorescence quenching: pyrene monomer and excimer emissions as sensors for electron deficient molecules, *Photochem. Photobiol. Sci* 4(10) (2005) 817–821. [PubMed: 16189557]
- [52]. Bains GK, Kim SH, Sorin EJ, Narayanaswami V, The Extent of Pyrene Excimer Fluorescence Emission Is a Reflector of Distance and Flexibility: Analysis of the Segment Linking the LDL Receptor-Binding and Tetramerization Domains of Apolipoprotein E3, *Biochemistry (Mosc)* 51(31) (2012) 6207–6219.
- [53]. Laskin DL, Sunil VR, Gardner CR, Laskin JD, Macrophages and Tissue Injury: Agents of Defense or Destruction?, *Annu. Rev. Pharmacol. Toxicol* 51(1) (2011) 267–288. [PubMed: 20887196]
- [54]. Sadtler K, Estrellas K, Allen BW, Wolf MT, Fan HN, Tam AJ, Patel CH, Luber BS, Wang H, Wagner KR, Powell JD, Housseau F, Pardoll DM, Elisseff JH, Developing a pro-regenerative biomaterial scaffold microenvironment requires T helper 2 cells, *Science* 352(6283) (2016) 366–370. [PubMed: 27081073]
- [55]. Bencherif SA, Sands RW, Ali OA, Li WA, Lewin SA, Braschler TM, Shih TY, Verbeke CS, Bhatta D, Dranoff G, Mooney DJ, Injectable cryogel-based whole-cell cancer vaccines, *Nat. Commun* 6 (2015).
- [56]. Anderson J, McNally A, Biocompatibility of implants: lymphocyte/macrophage interactions, *Semin Immunopathol* 33(3) (2011) 221–233. [PubMed: 21271251]
- [57]. Jiang K, Weaver JD, Li Y, Chen X, Liang J, Stabler CL, Local release of dexamethasone from macroporous scaffolds accelerates islet transplant engraftment by promotion of anti-inflammatory M2 macrophages, *Biomaterials* 114 (2017) 71–81. [PubMed: 27846404]
- [58]. Novak ML, Koh TJ, Phenotypic Transitions of Macrophages Orchestrate Tissue Repair, *Am. J. Pathol* 183(5) (2013) 1352–1363. [PubMed: 24091222]

- [59]. Spiller KL, Nassiri S, Witherel CE, Anfang RR, Ng J, Nakazawa KR, Yu T, Vunjak-Novakovic G, Sequential delivery of immunomodulatory cytokines to facilitate the M1-to-M2 transition of macrophages and enhance vascularization of bone scaffolds, *Biomaterials* 37 (2015) 194–207. [PubMed: 25453950]
- [60]. Oliveira MI, Santos SG, Oliveira MJ, Torres AL, Barbosa MA, Chitosan drives anti-inflammatory macrophage polarisation and pro-inflammatory dendritic cell stimulation, *Eur Cell Mater* 24 (2012) 136–153. [PubMed: 22828991]
- [61]. Stables MJ, Shah S, Camon EB, Lovering RC, Newson J, Bystrom J, Farrow S, Gilroy DW, Transcriptomic analyses of murine resolution-phase macrophages, *Blood* 118(26) (2011) E192–E208. [PubMed: 22012065]
- [62]. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, Sharpe AH, PD-L1 regulates the development, maintenance, and function of induced regulatory T cells, *J. Exp. Med* 206(13) (2009) 3015–3029. [PubMed: 20008522]
- [63]. Wynn TA, Vannella KM, Macrophages in Tissue Repair, Regeneration, and Fibrosis, *Immunity* 44(3) (2016) 450–462. [PubMed: 26982353]
- [64]. Wang J, Cao Z, Zhang X-M, Nakamura M, Sun M, Hartman J, Harris RA, Sun Y, Cao Y, Novel Mechanism of Macrophage-Mediated Metastasis Revealed in a Zebrafish Model of Tumor Development, *Cancer Res* 75(2) (2015) 306–315. [PubMed: 25492861]
- [65]. Vacanti NM, Cheng H, Hill PS, Guerreiro JDT, Dang TT, Ma M, Watson S, Hwang NS, Langer R, Anderson DG, Localized Delivery of Dexamethasone from Electrospun Fibers Reduces the Foreign Body Response, *Biomacromolecules* 13(10) (2012) 3031–3038. [PubMed: 22920794]
- [66]. Graham DK, DeRyckere D, Davies KD, Earp HS, The TAM family: phosphatidylserine-sensing receptor tyrosine kinases gone awry in cancer, *Nat. Rev. Cancer* 14 (2014) 769. [PubMed: 25568918]
- [67]. Birge RB, Boeltz S, Kumar S, Carlson J, Wanderley J, Calianese D, Barcinski M, Brekken RA, Huang X, Hutchins JT, Freimark B, Empig C, Mercer J, Schroit AJ, Schett G, Herrmann M, Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer, *Cell Death Differ* 23(6) (2016) 962–978. [PubMed: 26915293]

Fig. 1.
Schematic illustration of macroporous red blood cell membrane-derived (RBCM) scaffold and its fabrication.

Fig. 2.

Characterization of RBCM vesicles and RBCM scaffolds. A) Size distribution of RBCM vesicles measured by DLS. B) A representative TEM image of RBCM vesicles. Scale bar, 200 nm. C) An RBCM scaffold labeled with RhB-DMPE for visualization. D) A representative SEM image of RBCM scaffolds. Scale bar, 100 μ m. E) Representative confocal microscopy images of RBCM scaffolds. FITC-BSA was added to label the interconnected frame structure, and RBCM vesicles were labeled with RhB-DMPE. Scale bar for overlay, 200 μ m; scale bar for higher magnification, 50 μ m. F) Effective indentation

modulus of RBCM and MA-alginate scaffolds. G) Effective indentation modulus of RBCM scaffolds with 20 μ L of alginate (2 wt%) and various amounts of RBCM vesicles. The ratio is the weight ratio of vesicles and alginate. Values are presented as mean $\pm$ SD (n = 3). * p < 0.05, ** p < 0.01.

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Fig. 3.

Homogeneous dispersion of hydrophobic model drug pyrene in RBCM scaffolds and its sustained-release from RBCM scaffolds. A) Representative emission spectra of pyrene in RBCM vesicle solution and PBS with an excitation at 320 nm. B) The ratio of fluorescence intensity at 390 nm and 456 nm of pyrene in RBCM vesicle solution and PBS. C) Representative emission spectra of pyrene in RBCM and MA-alginate scaffolds with an excitation at 320 nm. D) The ratio of fluorescence intensity at 390 nm and 456 nm of pyrene in RBCM scaffolds and MA-alginate scaffolds. E) The release of pyrene from RBCM

scaffolds in PBS with 10% FBS and 1% PS at 37 °C and 100 × rpm of shaking. Values are presented as mean ± SD (n = 3). *** $p < 0.001$, **** $p < 0.0001$.

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Fig. 4.

In vivo characterization of cell infiltration into RBCM and MA-alginate scaffolds. A) Cell numbers in RBCM and MA-alginate scaffolds on day 1, 4, and 10 after s.c. injection of scaffolds in mice. B) Percentages and numbers of dendritic cells, neutrophils, and macrophages in RBCM and MA-alginate scaffolds after scaffold injection. C) Representative flow cytometry plots of macrophages in RBCM and MA-alginate scaffolds after 10 days with F4/80 as X axis and side scatter as Y axis. Values are presented as mean $\pm$ SD (n = 3). * $p < 0.05$.

Fig. 5.

Characterization of macrophages in RBCM and MA-alginate scaffolds and M0, M1, and M2 BMDMs. A) qPCR analysis of M2 gene marker Arg-1 and M1 gene marker iNOS expression in cells from RBCM and MA-alginate scaffolds and M0, M1, and M2 BMDMs. B) Percentages of MHC-II⁺ cells and MFI of MHC-II among F4/80⁺ cells in RBCM and MA-alginate scaffolds and M0, M1, and M2 BMDMs. C) Percentages of PD-L1⁺ cells and MFI of PD-L1 among F4/80⁺ cells in RBCM and MA-alginate scaffolds and M0, M1, and M2 BMDMs. Values are presented as mean $\pm$ SD (n = 3) (from two independent experiments). * p < 0.05, ** p < 0.01.

Fig. 6.

Biocompatibility study of RBCM and MA-alginate scaffolds. A) *In vivo* concentrations of IL-10 and IL-12 in RBCM and MA-alginate scaffolds on day 4 and in spleens, liver, and lymph nodes. The concentrations were measured by ELISA. Values are presented as mean $\pm$ SD (n = 3), * p < 0.05, ** p < 0.01 versus spleen, liver, and LN. B) H&E staining of RBCM scaffolds and MA-alginate scaffolds 2 weeks and 1 month after their s.c. injections in the backs of C57BL/6J mice. Black dash lines delineate fibrotic capsule layers. Scale bar, 100 μ m.

Fig. 7.

In vivo uptake of RBCM vesicles from RBCM scaffolds by infiltrated macrophages. A) A representative confocal image of Alexa Fluor 647-labeled RBCs. Scale bar = 50 μ m; Inset scale bar = 10 μ m. B) Percentages of dendritic cells, neutrophils, and macrophages in Alexa Fluor 647-labeled RBCM scaffolds after scaffold injection. Values are presented as mean $\pm$ SD (n = 3), * p < 0.05. C) Histogram plots of RBCM vesicle uptake by macrophages at various time points. D) The MFI of macrophages in scaffolds internalizing RBCM vesicles at various time points. Values are presented as mean $\pm$ SD (n = 3), * p < 0.05, ** p < 0.01.