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Macrophage phenotypic mechanomodulation of enhancing bone regeneration by superparamagnetic scaffold upon magnetization

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ABSTRACT

Macrophages are involved in all phases of scaffold induced tissue regeneration, orchestrating the transition from an inflammatory to regenerative phenotype to guide all other cell types to complete the wound healing process when a tissue defect advances beyond the critical size. Therefore, harnessing macrophages by scaffolds is important for facilitating tissue regeneration *in situ*. In this work we utilized the superparamagnetic scaffold upon magnetization as a mechanostimulation platform to apply forces directly to macrophages grown in the scaffold, aiming to figure out whether the functions of macrophages related to bone tissue regeneration can be mechanomodulated and to elucidate the underlying mechanisms. We showed the first evidence that upon magnetization the interaction of superparamagnetic scaffolds to macrophages drove them to polarize towards an M2-like phenotype by inhibiting TLR2/4 activation and enhancing VEGFR2 activation, thereby inhibiting secretion of the pro-inflammatory cytokines IL-1β, TNF-α and MCP-1, as well as the osteoclast differentiation cytokines MMP-9 and TRAP, and up-regulating VEGF and PDGF. The conditioned media enhanced the osteogenesis of osteoblasts and the angiogenesis of endothelial cells.

1. Introduction

Macrophages are involved in all phases of the natural process of wound healing [1–3]. One primary method by which macrophages regulate tissue regeneration is cytokine secretion. Macrophages can be polarized differently, depending on their environment, to secrete various cytokines, including interleukins, chemokines, and growth factors, as well as other small molecules [4]. In this way, macrophages orchestrate the transition from an inflammatory to regenerative phenotype, guiding all other cell types to work together to complete the wound healing process [5–7]. However, when a tissue defect advances beyond the critical size, a scaffold has to be implanted *in situ* to guide tissue regeneration and repair the defect, as wound healing does not occur naturally. In contrast to the natural process, implanted scaffolds generally cause long-term inflammation due to the foreign body reaction (FBR) initiated by macrophages [8], one of the first populations to arrive after implantation; severe and/or continuous inflammation can impede healing [9–12]. Thus, the responses of macrophages encountering the implanted scaffold can, to a large extent, determine whether scaffold-guided tissue repair is successful. Therefore, harnessing macrophages by scaffolds is important for facilitating tissue regeneration *in situ*.

We previously reported that superparamagnetic scaffolds applied interactions to pre-osteoblasts under a magnetic field (MF) to enhance osteogenesis *in vitro* [13] and *in vivo* [14], because the scaffold can respond to an applied MF to be magnetized. Recently, accumulating evidence has demonstrated that the combination of superparamagnetic scaffolds and MFs could have a positive influence on many tissue-specific cell types including osteoblasts [15,16], cartilage cells [17], endothelial cells [18,19], Schwann cells [20] and fibroblasts [21], and stem cells [22]. However, macrophages have not been examined in this regard. Therefore, the aim of

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this study was to investigate whether the functions of macrophages related to bone tissue regeneration can be modulated using a superparamagnetic scaffold upon magnetization and to elucidate the underlying mechanisms. Here, we provide the first evidence that the combination of magnetic responsive scaffold with MF modulated the function of macrophages, which was benefit to the angiogenesis and osteogenesis.

2. Materials and methods

2.1. Preparation and characterization of composite scaffolds

Magnetic γ-Fe_2O_3 nanoparticles (7–10 nm in core diameter) with a coating of meso-2, 3-dimercaptosuccinic acid (MNPs) were provided by the Nanjing Nanoeast Biotech Co., Ltd. Magnetic nanofibrous scaffolds (mag-S) were prepared according to the following procedure: 0.1 g–0.25 g of MNPs and 1 g of hydroxyapatite nanoparticles (nHA, 20 nm in average diameter, Nanjing Emperor Nano Material Co., Ltd, Nanjing, China) were dispersed in 10 mL of *N*,*N*-dimethyl acetamide (DMAc) by aid of probe sonication to form a homogenous suspension. Then 2 g of poly lactide (PLA, average molecular weight is 80 kD, Chang Chun Sino Biomaterial Co., Ltd, Changchun, China) was dissolved in the suspension. The resulting solution was subjected to an electrospinning device and processed with the following optimum parameters: needle inner diameter was 0.9 mm, distance between the needle's tip and the collector was 25 cm, and voltage was 15 kV [13]. As control, two-component composite scaffold of PLA and nHA (named as con-S) was prepared using the similar procedure.

The super-paramagnetic property of the mag-S was measured with the vibrating sample magnetometer (VSM, Lakeshore 7407). The morphology of mag-S and con-S was observed by scanning electron microscope (SEM, Hitachi s-4800). For uses in the cell culture, mag-S and con-S were spread in polyurethane substrate film and trimmed to fit the well of cell culture plates. All samples for cell and animal experiments were sterilized with ethylene oxide (SATOU, HMQ-78L). The endotoxin levels in all samples were detected below 0.1 EU/mL (ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit, GenScript, Nanjing China).

2.2. Static magnetic field setting in the cell culture system and cells

One permanent magnet was fixed beneath the cell culture plate, building the magnetic field (MF) in the wells. By adjusting the distance between the magnet and the culture plate, the different strength of MF was set in separate areas (Fig. S1), which was measured using a Digital Teslameter (SG-4L, No.14003). In order to make a dynamic-like stimulation, the magnetic field was applied alternatively in an interval of 12 h incubation.

Murine monocyte macrophage cell line (RAW264.7) and pre-osteoblast cell (MC3T3-E1) was purchased from the Cell Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). RAW264.7 was maintained in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 4 mM of L-glutamine, 4500 mg/L glucose, and 0.1% penicillin G and streptomycin at 37 °C with 5% CO_2. MC3T3-E1 was maintained in α-modified Eagle's medium (α-MEM) and supplements. Primary human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell Research Laboratories (San Diego, CA), and cultured in endothelial cell medium with growth supplements provided by the company.

2.3. Cell proliferation assay (CCK-8 assay)

RAW264.7 of 1×10^4 was seeded on mag-S and con-S with different magnetic fields strength at 12 h intervals. After 24, 48 and 72 h of incubation, 100 μL of fresh medium containing Cell Counting Kit 8 reagent (CCK-8; Dojindo) was added to each well and incubated for another 1 h at 37 °C. The supernatants of each sample were aspirated and the absorbance was measured at 450 nm using a microplate reader (Epoch R, BioTek).

2.4. Cytokines expression assay

RAW264.7 of 2×10^5 was seeded on mag-S and con-S in a 12-well culture plate under magnetic fields. After 72 h-incubation, the supernatants were collected. Cell cytokines were quantified by corresponding assay kit and three replicates were set in each assay. Mouse interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), vascular endothelial growth factor (VEGF) ELISA kit were purchase from eBioscience. Monocyte chemoattractant protein-1 (MCP-1) ELISA kit was purchased from Neobioscience. Platelet-derived growth factor-BB ELISA kit (PDGF-BB) was purchased from Cusabio. Matrix metalloprotein 9 (MMP9) ELISA kit was purchase from R&D. The tartrate resistant acid phosphatase (TRAP) amount was detected by specific assay kit (Beyotime) according to the manufacturer's instruction. The concentrations of the cytokines were normalized to the cell number in each group.

2.5. Western blot

RAW264.7 of 2×10^5 was seeded on mag-S and con-S in a 12-well culture plate. After 72 h-incubation, the cells were washed with PBS and lysed in RIPA cell lysis buffer (Beyotime Biotechnology, Haimen, China) supplemented with protease inhibitor (PMSF, Sigma-Aldrich) on the ice for 30 min. The lysates were clarified by centrifugation at 12000 rpm for 15 min. Protein concentration of the lysates was determined by a Pierce BCA protein assay kit (Pierce Biotechnology, Rockford, IL). After denatured by boiling in loading buffer (Transgen Biotech, China), equal amount of protein (20 μg) was loaded and separated on 10% polyacrylamide gels (Applygen, Beijing China) and transferred to poly(-vinylidenedifluoride) membranes (PVDF, 0.45 μm; Millipore, Bedford, MA). Primary antibody to arginase-1 (Arg1), inducible nitric oxide synthase (iNOS) (Abcam), toll-like receptor 2 (TLR2), TLR4, vascular endothelial growth factor receptor 2 (VEGFR2), nuclear factor NF-κB, hypoxia-inducible factor 1α (HIF-1α) (Cell Signaling technology) and β-actin (Sigma-Aldrich) was applied to probe corresponding protein expression. After three washes in Tris buffered solution (TBST), the membrane was incubated in secondary antibody conjugated to horseradish peroxidase (HRP) (Zhongshan Goldenbridge biotechnology Co, Beijing, China) for 2 h at room temperature. After washes, the immunocomplex on the membrane was visualized using Image QuantLAS 4000 (GE Healthcare) with Immobilon Western HRP Substrate luminol reagent and peroxide solution (Millipore, Billerica, MA).

2.6. Confocal observation of actin

The mag-S was spread on round coverslips and placed in the 24-well plate. RAW264.7 cells were seeded on the coverslips at a density of 8×10^4 cells/well and incubated overnight to allow adherence. The medium was replaced with 0.5 mL of fresh medium or fresh medium containing 50 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich), followed by 72 h-incubation under magnetic fields. After that the cells were rinsed twice with phosphate buffered solution (PBS; pH = 7.4) and fixed with 4% formaldehyde in PBS for