Characterizing Immune Cell Infiltration During Osteochondral Injury and Biomaterial Implantation

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INTRODUCTION:
Osteochondral (OC) defects disrupt the articular cartilage and underlying bone, subsequently inducing an inflammatory wound healing response that can result in fibrocartilage repair[1]. The immune response is increasingly being considered pivotal in tissue repair, and it is therefore hypothesized that identification of infiltrating immune cells will provide insight on OC tissue repair. Rat leukocyte identification by flow cytometry is relatively understudied but may be crucial to determine the immune response within the injury space during the wound-healing process. The overall goal of this research is to develop an OC-mimetic composite for *in vivo* OC tissue repair. We have developed a composite scaffold using a stiff, 3D-printed multilayer structure that can be infilled with tissue-mimetic hydrogels and implanted to study the native host immune response in the context of osteochondral tissue repair. The specific goals of this study are to (a) establish an OC-mimetic composite for rat OC injury, and (b) study immune cell infiltration in the osteochondral defect space with and without the OC-mimetic composite.
METHODS: *3D Printing:* Photo-resin was prepared by mixing PEGDA 700 and PETMP (99:1 wt%) with 0.85 wt% TPO as a photoinitiator, 0.05 wt% of AIBN as a thermal initiator, and 0.8 wt% Tinuvin CarboProtect® as a photoabsorber, and used to fabricate the 3D printed structures[2] at λ = 405 nm. After printing, the structures were rinsed and thermally cured in an oven at 120ºC . Bilayer structures had an average dimension of 2 mm in height and 2 mm in diameter. For cell studies, the structures were sterilized in 70% ethanol for a minimum of two hours. *Fabrication of the Soft Biomimetic Hydrogels:* TGF-β3 was reacted with a four-molar excess of 2-iminothiolane for one hour at room temperature to produce TGFβ3-SH[3]. Thiolated growth factor was tethered to 8-arm-10kDa PEG-norbornene (PEGNB) via thiol-norbornene photoclick chemistry at a concentration of 50 nM TGFβ3-SH. The soft cartilage-mimetic hydrogel (CMH) was formed from the following formulations: a prepolymer solution of 9 wt% PEGNB, MMP-sensitive crosslinker (GCVPLS-LYSGCG, 0.97:1 thiol:ene), GCRGDS (0.1 mM), thiolated chondroitin sulfate (1 wt%), and photoinitiator LAP (0.05 wt%). *Animal Studies:* Lewis rats (11 weeks old, female) received a critical size OC defect created in the trochlear groove of the knee using a 2mm diameter bur to a depth of 2mm. For the composite scaffold, gel precursor solution was injected into the 3D printed structure and then photopolymerized *in situ* with 405 nm light at 200 mW cm^{-2} for 1 minute. At 7 days post-implantation, acellular composite scaffold samples in the femur were fixed, GMA-embedded, and stained for nuclei and proteoglycans. A separate study analyzed native cell infiltration to an OC defect over 14 days post-injury. Cells from the defect space were isolated, stained for viability, CD45, CD3, CD45R, CD43, CD68, and HIS48, and analyzed with flow cytometry.
RESULTS:
Bilayer (articular cartilage (AC) and subchondral bone (SB)) structures were printed with a custom DLP system and infilled with cartilage-mimetic (E=40 kPa) hydrogel for use in a pilot study to establish feasibility of a biomimetic composite for OC tissue repair in a rat model (Fig 2a). For acellular studies, composite bilayer scaffolds were implanted in a rat knee femoral osteochondral defect. Safranin-O/Fast green staining of a sagittal section of a femur (11 wk old, female, Lewis rat) revealed that native cells readily infiltrate into the AC and SB layers within 7 days (Fig 2b). Preliminary studies of native cell infiltration to the OC defect space highlight large populations of neutrophils and T cells within the injury space over two weeks (Fig 2c,d).
DISCUSSION:
A bilayer structure was 3D-printed using a PEGDA-based resin and infilled with a cartilage-mimetic hydrogel to serve as an interpositional material for rat osteochondral tissue repair. *In vivo*, the composite scaffold recruited native cells to infiltrate within 7 days. Preliminary flow cytometry analysis suggests large myeloid cell populations within the defect space. Taken together, this suggests that innate and adaptive immune cells are present and recruited to the site of injury where they can enter and interact with the hydrogel microenvironment. Further work will incorporate in-depth characterization of monocyte and macrophage populations in response to the implanted biomimetic hydrogel.
SIGNIFICANCE:
This work demonstrates a proof-of-concept model for studying osteochondral injury and tissue repair in a rat model with an OC-mimetic composite. The tunable hydrogel system allows for the incorporation of bioactive cues that can influence recruitment of native immune cells to the defect site. Development of a robust flow cytometry panel to identify rat leukocyte populations will allow for temporal tracking of the host immune response in response to composite implantation.
REFERENCES: 1. M. Li *et al.*, *Acta Biomaterialia*, vol. 140, pp. 23–42, Mar. 2022. **2.** Aisenbrey, E. A. *et al.*, Macromolecular Bioscience 18 (2), 1700267**. 3.** Sridhar, B. V. *et al.*, J. Biomed. Mater. Res., Part A. 2014, pp 4464–4472.
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IMAGES:

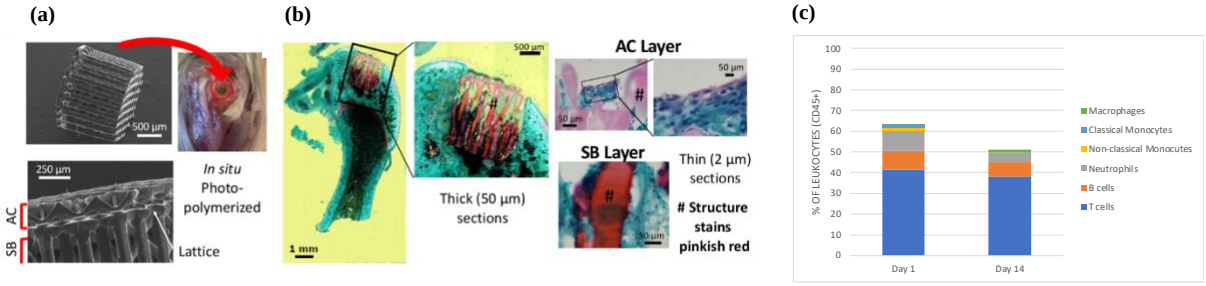

Figure 1: Rat OC defect pilot study. (a) SEM image of the 3D printed structure with AC and SB layers. **(b)** Safranin-O/Fast green stain of the sagittal section of an OC defect filled with the composite scaffold (structure + acellular CMH) 7d post-implantation. Shown is the 3D structure location (#) at increasing magnification. **(c)** Bar graphs of leukocyte populations within the femoral defect space at 1 day and 14 days post-injury.