Research Article

The cellular responses of human macrophages seeded on 3D printed thermoplastic polyurethane scaffold

3B Baskılı termoplastik poliüretan iskeleye ekilen insan makrofajlarının hücresel yanıtları

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Abstract: Tissue engineering is an interdisciplinary field for the design of functional constructs that aid to repair damaged or diseased tissue. Three-dimensional (3D) printing is a growing technology that offers new opportunities for tissue engineering. Thermoplastic polyurethane (TPU) is a member of the polyurethane class. TPUs are commonly used in medical applications with their biocompatible, superior mechanical properties and shape memory behavior. Macrophages are key regulators of tissue homeostasis, inflammation, and regeneration. They play crucial roles in initial immune response to implants. In this study, we aimed to investigate the viability, adhesion, and distribution properties of human THP-1 macrophages seeded on 3D printed TPU scaffolds in vitro. The expression of CD68 and CD10 was also analyzed in human THP-1 macrophages on 3D TPU scaffolds. THP-1 macrophages treated with phorbol-12-myristate-13-acetate (PMA) were seeded on 3D TPU scaffolds or tissue culture plastic plates as control and cultured for 1, 3, 7, and 14 days. 3D TPU scaffolds were prepared using a custom made fused deposition modeling printer. The cell viability was measured by WST-1 assay on days 1 and 3. The cell adhesion was evaluated by scanning electron microscopy (SEM). The cell distribution was analyzed by hematoxylin and eosin (H&E) staining. Expression of CD10 and CD68 was analyzed by immunohistochemical (IHC) staining. The viability of THP-1 macrophages on 3D TPU scaffolds was lower than their control groups on days 1 and 3. SEM images showed THP-1 macrophage attachment on the 3D TPU scaffold surface with round and elongated morphologies. H&E staining demonstrated that THP-1 macrophages showed eosinophilic cytoplasm and large nuclei. CD68 staining was more intense in THP-1 macrophages on 3D TPU scaffolds on day 3 compared to days 1, 7 and 14. CD10 staining was more intense on day 1 compared to days 3, 7, and 14. Our results show that 3D TPU scaffolds are biocompatible with macrophages and might be a potential biomaterial for medical applications.

Keywords: macrophages, 3D printed, TPU, cell culture

Özet: Doku mühendisliği, hasarlı veya hastalıklı dokuyu onarmak için destekleyici fonksiyonel yapıların tasarımı için disiplinler arası bir alandır. Üç boyutlu (3B) baskı, doku mühendisliği için yeni fırsatlar sunan ve büyümekte olan bir teknolojidir. Termoplastik poliüretan (TPU), poliüretan sınıfının bir üyesidir. TPU'lar, biyouyumlulukları, üstün mekanik özellikleri ve şekil hafızalı davranışları ile tıbbi uygulamalarda yaygın olarak kullanılmaktadır. Makrofajlar, doku homeostazı, inflamasyon ve rejenerasyonun anahtar düzenleyicileridir. İmplantlara karşı ilk bağışıklık yanıtında çok önemli rol oynarlar. Bu çalışmada, 3B baskılı TPU doku iskelelerine ekilen insan THP-1 makrofajlarının canlılık, adezyon ve dağılım özelliklerini in vitro olarak araştırmayı amaçladık. Ayrıca, 3B TPU doku iskelelerine ekilen insan THP-1 makrofajlarında CD68 ve CD10'un ekspresyonu da analiz edildi. Forbol-12-miristat-13-asetat (PMA) ile uyarılan THP-1 makrofajları, 3B TPU doku iskeleleri ve kontrol olarak doku kültürü plastik plakaları üzerine ekildi ve 1, 3, 7 ve 14 gün boyunca kültüre edildi. 3B TPU doku iskeleleri, özel yapılmış bir eriyik yığma modelleme (FDM) yazıcısı kullanılarak hazırlandı. Hücre canlılığı, 1. ve 3. günlerde WST-1 kiti ile ölçüldü. Hücre adezyonu, taramalı elektron mikroskobu (SEM) ile değerlendirildi. Hücre dağılımı, hematoksilen ve eozin (H&E) boyaması ile analiz edildi. CD68 ve CD10 ekspresyonu, immünohistokimyasal (IHC) boyama ile analiz edildi. THP-1 makrofajlarının 3B TPU doku iskeleleri üzerindeki canlılığı, 1. ve 3. günlerde kontrol gruplarından daha düşük tespit edildi. SEM görüntüleri, THP-1 makrofajların 3B TPU doku iskeleleri yüzeyinde yuvarlak ve uzun morfolojilere sahip olarak tutunduğunu gösterdi. H&E boyaması ile THP-1 makrofajlarının eozinofilik sitoplazma ve büyük çekirdekli morfolojide olduğu gösterildi. 3B TPU doku iskelelerindeki THP-1 makrofajların 3. günde CD68 boyaması 1, 7 ve 14. günlere kıyasla daha yoğundu. CD10 boyaması 1. günde 3, 7 ve 14. günlere kıyasla daha yoğundu. Sonuçlarımız, 3B TPU doku iskelelerinin makrofajlarla biyolojik olarak uyumlu olduğunu ve tıbbi uygulamalar için potansiyel bir biyomateryal olabileceğini göstermektedir.

Anahtar Kelimeler: makrofaj, 3D Baskı, TPU, Hücre Kültürü

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1.Introduction

Tissue engineering is an interdisciplinary field of engineering and life sciences for the development of functional constructs that restore, maintain, or improve damaged tissues or whole organs (1). Substantial interest in three-dimensional (3D) printable biopolymers for applications, such as scaffolds in tissue engineering, drug delivery devices, as well as artificial organs for surgery trainings, are constantly growing.

3D-printed tissue engineering constructs are used to provide a biomimetic structural environment that facilitates tissue formation and promotes host tissue integration (2).

Thermoplastic polyurethanes (TPUs) are linear segmented block polymers containing polar hard segments and relatively nonpolar soft segments with crystalline and amorphous domains. This molecular architecture allows TPUs with an excellent stretchability and viscoelastic behavior (3). They are used in the field of medical applications particularly in flexible uses such as blood vessels, and catheters, as well as wound dressings. They are characterized by high biocompatibility, biodegradability, adequate bending strength and resistance to abrasion (4). Moreover, properly designed thermoplastic polyurethanes (TPUs) are suitable materials for use in 3D printing by the Fused Deposition Modeling (FDM) method [5].

The success of tissue engineering is strongly related to the inflammatory response. Implantation of biomaterials stimulates host responses aiming at eliminating the implants as foreign objects. Monocytes are crucial in this host inflammatory and foreign body reaction to biomaterials. An inflammatory response is initiated and monocytes migrate to the tissue–material interface (6). Once attached to the surface of the implant, they mature into a macrophage phenotype. Macrophages are key cells in the initial immune response to implants. They also regulate the recruitment, proliferation and differentiation of other types of cells including fibroblasts, endothelial cells, keratinocytes (7). They determine whether the inflammatory process subsides or a fibrous capsule forms and thus whether tissue regeneration occurs.

A profound understanding of how biomaterials control inflammatory response is important for the development of implants In particular, modulating the macrophage responses is of interest due to its relationship with not only the wound repair process, but also the foreign body response. The aim of this study is to investigate the viability, adhesion, distribution and CD68 and CD10 expression of human THP-1 macrophageses seeded on 3D printed TPU scaffolds in vitro.

2.Materials and Methods

Cell culture

The human monocytic leukemia cell line THP-1 was obtained from DSMZ (Germany). THP-1 cells were cultured using RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified 5% CO2 atmosphere. Cells were used for experiments between passage numbers 8 and 10. For differentiation to a macrophage phenotype, THP-1 cells were incubated with 100 nM of phorbol-12-myristate-13-acetate (PMA) (Applichem, Germany).

Fabrication of 3D Scaffolds

Scaffolds template (Ø = 4 mm, thickness = 2 mm) were designed using SolidWorks 2019 software and subsequently filled and sliced using and Ultimaker Cura 4.11 software to obtain cylindrical STL models. Briefly, clump generator software was used to create squared pores into a 3D object in a “stl” □le format. 3D-printed scaffolds were prepared from TPU (eSun Filament, Shenzhen, China). The 3D printer was a customized system working by the mechanism of FDM. Printing was performed by the custom made FDM printer, using a nozzle diameter of 0.4 mm, a layer thickness of 0.2 mm, a nozzle temperature of 240 °C TPU, and a printing bed temperature of 40°C. All scaffolds had a thickness of 2 mm. Both layers were printed with three perimeter lines and rectilinear filling under an angle of 0-90°, applying a flow rate of 100%. Printing speed was set to be 60 mm/s for all materials. Prior to biological evaluations, printed scaffolds were sterilized by ethylene oxide.

Cell Seeding on 3D Scaffolds

Sterile 3D TPU scaffolds were immersed in complete medium in the 96-well plate and incubated overnight in a humidified incubator (37°C, 5% CO2) prior to cell seeding. Then, suspension of THP-1 monocytes in complete medium with 100 nM PMA were seeded on the scaffolds at 4x104 cells per well and incubated in the same incubator for 4 h to allow cell attachment. Each well was completed to 150 µL complete medium in total volume. In order to eliminate the cells that do not attach to scaffolds, 16-24 hours after seeding the scaffolds were placed in another 96-well plate. THP-1 macrophages with equivalent numbers were also seeded on tissue culture plastic plates without scaffolds as a control group. 3D TPU scaffolds were cultured for 1 and 3 days.

Measurement of THP-1 macrophages viability on 3D TPU Scaffolds

The viability of THP-1 macrophages on 3D TPU scaffolds was determined by ready-to-use colorimetric WST-1 assay (Biovision, Milpitas, CA, USA) on days 1 and 3 after seeding of the cells. The assay protocol is based upon the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. The amount of formazan dye produced was measured at 450 nm by using a microplate reader (Biotek Synergy HTX, USA). The cell viability was expressed in percentage relative to a control group.

Scanning electron microscopy

After seeding, THP-1 macrophages were cultured for 1 and 3 days on 3D TPU scaffolds. Then, they were fixed to evaluate the adhesion and the morphology of cells by scanning electron microscopy (SEM). The cell-scaffolds constructs were transferred to the 24-well plate, fixed in 4% paraformaldehyde, dehydrated through a graded series of ethanol solutions (60%, 70%, 80% and 99% ethanol) (v/v). Then the scaffolds were wrapped in aluminum foil and dried in the desiccator for 2 days (8). The scaffolds placed on the platform were plated with 5 nm gold for 20 minutes by the Q150R S (Quorum) instrument. Copper banding was then applied to the platform to eliminate charging effect. Images were taken by ZEISS Sigma 500 VP FE-SEM.

Histological staining of examination

THP-1 macrophages on 3D TPU scaffolds were cultured for 1, 3, 7, and 14 days. Cell-scaffold constructs were removed, washed three times in PBS and fixed in 4% paraformaldehyde at 4 °C for 24 h. This was followed by a tissue processing procedure, embedded in paraffin and cut into 5 µm thick sections. The sections were then stained with hematoxylin & eosin (H&E). The morphology and distribution of THP-1 macrophages cultured on 3D TPU scaffolds were examined under light microscope (Olympus, BX51 microscope) (9). In immunohistochemical (IHC) evaluation, 5 µm thick sections were treated with CD68 (bs-0649R, Bioss) and CD10 (bs-0709R, Bioss) antibodies and incubated at 4°C for overnight. Antigenic sites were visualized by diaminobenzidine solution and counterstained with hematoxylin. The images were taken with a light microscope (Olympus BX51 microscope) with X20 magnification (10, 11).

Statistical analysis

Statistical analyses were performed using SPSS 24 software program. The results were expressed as mean ± SD. Two groups were compared using Mann Whitney U test. A value of $p<0.05$ was considered significant.

3. Results

The viability of THP-1 macrophages on 3D TPU Scaffolds

Compared to the control cells seeded on tissue culture plastic plates without scaffolds, the number of THP-1 macrophages residing in the 3D TPU scaffolds showed a decrease on day 1 (p □ 0,05). The number of THP-1 macrophages on 3D TPU scaffolds showed the same trend on day 3 (p<0,05) (Figure 1).

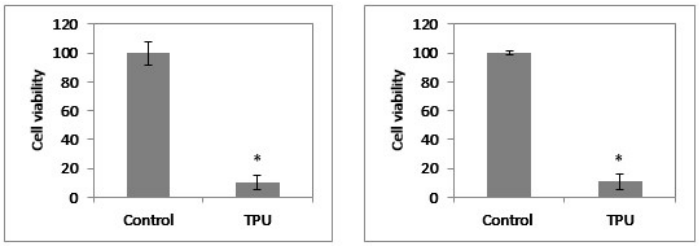

Figure 1. The viability of THP-1 macrophages on 3D TPU scaffolds on days 1 and 3.

The morphology of THP-1 macrophages on 3D TPU scaffolds

We investigated whether THP-1 macrophages were capable of adhering to 3D TPU scaffolds and vizualized via SEM. The cell distribution was homogeneous and THP-1 macrophages adhered to the surfaces of 3D TPU scaffolds on days 1 and 3. The cells were found to adhere to the scaffolds both in single cells and interacting cells. They showed a more rounded morphology on day 1 and a more elongated morphology on day 3 (Figure 2).

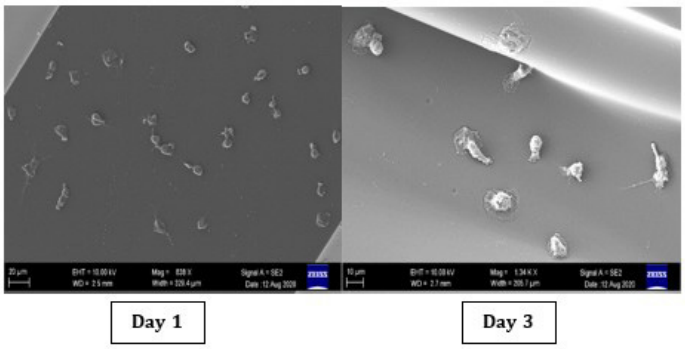

Figure 2. SEM images of THP-1 macrophages on 3D TPU scaffolds on days 1 and 3.

Histological analysis

THP-1 macrophages seeded on 3D TPU scaffolds showed tissue spesific morphologies in H&E staining on days 1, 3, 7, and 14. They were found to have eosinophilic cytoplasm and large nuclei. The distribution of cells on 3D TPU scaffolds were similar on all four days (Figure 3).

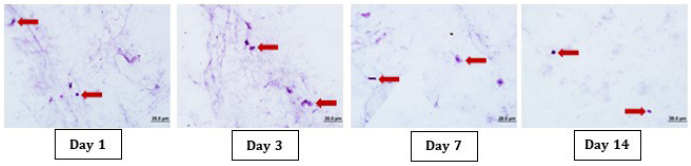

Figure 3. H&E staining in THP-1 macrophages seeded on 3D TPU scaffolds on days 1, 3, 7, and 14. Red arrows indicate THP-1 macrophages.

THP-1 macrophages on 3D TPU scaffolds were stained with CD68 (pan macrophage) macrophage marker to visualize the distribution of macrophages. Immunoreactivity for CD68 was observed in THP-1 macrophages on days 1, 3, 7, and 14. CD68 expression was stronger on day 3 compared to other days. THP-1 macrophages on 3D TPU scaffolds were also stained with CD10 antibody. CD10 expression was observed in THP-1 macrophages on days 1, 3, 7, and 14. CD10 expression was stronger on day 1 compared to other days.

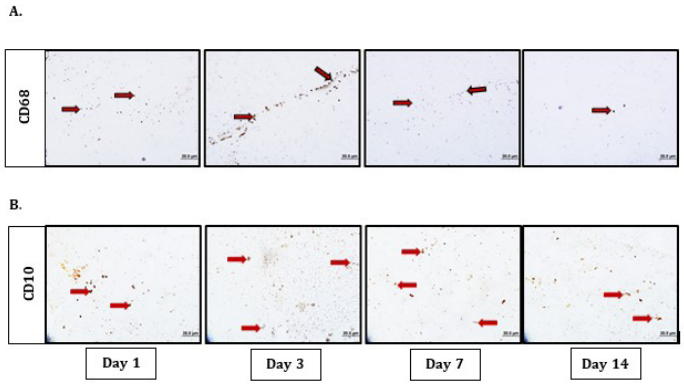

Figure 4. CD68 (A) and CD10 (B) staining in THP-1 macrophages seeded on 3D TPU scaffolds on days 1, 3, 7, and 14. Red arrows indicate CD68 (A) and CD10 (B) stained THP-1 macrophages.

4.Discussion

In our present study, we investigated the biocompatibility of 3D printed TPU scaffolds by using THP-1 monocyte-derived macrophages in vitro. THP-1 monocytes were treated with PMA and seeded on the 3D TPU scaffolds. Then, we analyzed the viability, adhesion. distribution and CD68 and CD10 expression of human THP-1 macrophages on the 3D TPU scaffolds.

Biomaterials are commonly used in medical applications. The application of 3D printing have greatly developed the manufacture of scaffolds in tissue engineering. TPU is a linear polymer that consists of hard and soft segments. They are used in medical applications, mainly because of its biocompatibility, high fracture strain, moderate tensile strength, and excellent abrasion and tear resistances. The soft segments provide elastomeric character, while hard segments usually provide additional strength (12).

The immune system is the first point of interaction between the body and the implant. It plays roles in biological processes required for the integration of biomaterials. Macrophages are instrumental in the host inflammatory and foreign body reaction to biomaterials. SEM images showed that THP-1 macrophages adhered to the surfaces of 3D printed TPU scaffolds on days 1 and 3 after seeding. This result showed the biocompatibility of 3D TPU scaffold with respect to THP-1 macrophages. The number of THP-1 macrophages on the 3D TPU scaffolds showed a decrease on days 1 and 3 compared to control group. We used 3D TPU scaffolds with soft properties in our study. A previous study showed that the number of 3T3 fibroblasts on soft TPU scaffolds were lower than hard TPU scaffolds (13). Woitschach et al reported that the number of human monocytes on soft TPU scaffolds were lower than hard TPU scaffolds (14). Our low cell viability results for TPU scaffolds are similar with these studies.

THP-1 macrophages seeded on 3D TPU scaffolds showed tissue spesific morphologies in H&E staining on days 1, 3, 7, and 14. They were found to have eosinophilic cytoplasm and large nuclei. CD68 is a pan macrophage marker (15). The presence of the macrophage CD68 positive THP-1 macrophages on 3D TPU scaffolds was evident on days 1, 3, 7, and 14. CD68 expression was stronger on day 3 compared to other days. CD10 is a cell surface neutral endopeptidase which is expressed by lymphocytes, neutrophils and monocytes (16). We observed CD10 expression in THP-1 macrophages on 3D TPU scaffolds on days 1, 3, 7, and 14. CD10 expression was stronger on day 1 compared to other days. A previous study showed that suspended THP-1 monocytes expressed no surface CD10 in the absence of PMA; however, after treatment with PMA, THP-1 cells differentiated to macrophages and CD10 was identified by flow cytometry (17). CD10 mRNA expression was also detected at 24 h and 72 h. Our result in consistence with this study

showed CD10 expression in THP-1 macrophages on 3D TPU scaffolds. Our histological observations demonstrated the presence and distribution of THP-1 macrophages on 3D TPU scaffolds.

5. Conclusion

In the present study, 3D printed TPU scaffolds was evaluated for the cellular responses of THP-1 macrophages in vitro. Our results provide data to better understand the immune responses and immunoengineering strategies using macrophages.

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