

Silk-based Matrices and C-kit Positive Cardiac Progenitor Cells for a Cardiac Organoid: Study of an in Vivo Model

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Abstract

In recent years, there has been a shift from tissue engineering to the production of organoids. The latter are useful tools to study many biochemical aspects and cellular reactions while avoiding the excessive use of laboratory animals. Organoids are very interesting tools because they can replicate the cellular and extracellular environment of an organ and retain some of the properties of the organ itself. However, without an adequate network of vessels, cell masses not only fail to grow, but they may exhibit an area of necrosis, indicating a lack of oxygen and nutrients. For this reason, scientific researchers are looking for ways to create organoids that can also mimic the vascular network of the organ from which they originate. One possibility is to implant the organoids in immunocompromised animals.

In the present study, we generated cardiac organoids *ex vivo* by seeding tyrosine protein kinase kit (c-kit)-positive cardiac progenitor cells (CPC cells) from fresh rat hearts into a rat collagen I gel. We then implanted these patches into immunosuppressed animals and compared the suitability of different silk fibroin scaffolds with three different geometries. We demonstrated that CPC cells were destroyed by CD3+ lymphocytes, that the porous and partially oriented scaffolds induced a consistent foreign body response compared to the electrospun meshes, and that CPC cells were degraded by a T-cell-mediated immune response, although the latter may be suitable for generating rat cardiac organoids.

1. Introduction

Tissue engineering has emerged in the last decade as an important approach to regenerate an injured organ by combining cells, matrices, biologically active molecules, and physiological stimuli. Cardiac tissue engineering can be used to restore cardiac function after surgery, for heart valve replacement, and to improve cardiac function after injury. In addition to controlling the biocompatibility and immunocompatibility of cell-seeded patches, it is important to develop methods to reduce the number of cells used for implantation and to identify scaffolds and pathways for cell differentiation [1].

Recently, an important aspect of an engineered tissue has emerged: it can be used as an organoid, a small defined environment similar to the *in vivo* organ, with the same properties, interactive and an important alternative to the use of animals in a research laboratory [2–3].

In the last few months, organoids consisting of 50% human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) and 50% nonmyocytes (with a 4:2:1 ratio of human ventricular cardiac fibroblasts (hCFs), human umbilical vein endothelial cells (HUVECs), and human adipose-derived mesenchymal stromal cells (hADSCs)) have been proposed as useful cardiac models to study toxicology, cell-cell interactions, genetic alterations, and novel therapies for cardiovascular disease [4–5].

Here we propose the use of tyrosine protein kinase kit (c-kit)-positive cardiac progenitor cells (CPCs) as the main population of a cardiac organoid. These cells were first identified in adult rat heart by Beltrami et al [6] and used as cell suspension in the first clinical trial of cardiac tissue regeneration [7]. In 2009, our research group showed that c-Kit-positive cardiac cells isolated from an adult rat heart and cultured in a

type I collagen gel were able to synthesize their own collagen fibers, organize vessels, form a capsule, differentiate into endothelial cells and cardiac fibroblasts, and differentiate cardiomyocytes [8]. In 2016, our research group demonstrated that porous silk fibroin scaffolds and electrospun nets can be used *in vitro* to induce rat adult CPC cell differentiation, and that partially oriented scaffolds were more efficient than the other materials tested. We also observed that partially oriented scaffolds and collagen alone were able to activate Z-body formation to initiate myofibril assembly [9].

Other three-dimensional (3D) structures have been used in the past to drive CPC cell differentiation. In an interesting study, using an experimental myocardial infarction model (MI) and injecting CPC cells, it was shown that the use of functionalized self-assembling peptide (SAP) hydrogels with a peptide mimicking the Notch 1 ligand, Jagged1 (RJ), improved acute retention and cardiac function [10].

In the present study, we isolated CPC cells from adult rat hearts, confirmed the expression of the three markers of multipotency (c-Kit, Sca-1 and MDR1) and Titin as a cardiac marker. Then, we tested their ability to differentiate in a 3D culture with collagen I *in vitro* and implanted these organoids in the subcutaneous (SC) region of immunocompromised animals [nude mice, severe combined immunodeficient (SCID) mice, and nude rats] to ensure their survival *in vivo* as well and to create a natural environment in which these organoids can implement their structure, complexity, and degree of differentiation. In parallel, we also investigated the suitability of silk fibroin scaffolds similar to those previously tested *in vitro* [9] and their potential use in creating this *in vivo* model of a cardiac organoid.

The results of our work showed that while CPC cells were able to express markers and structural proteins typical of cardiac muscle *in vitro*, *in vivo* they activated a CD3+ subset of T cells and caused their expansion (which was also present in nude animals despite their compromised immune system). The tested scaffolds alone induced giant cell activation and release of interleukin-4 (IL -4) and interleukin-13 (IL -13). Among the tested scaffolds, the fibroin electrospun nets (F-scaffolds) induced a lower response than the porous silk scaffolds previously used in our experiments to promote cardiac CPC differentiation *in vitro* [9]. Even though the electrospun fibroin electrospun nets induced a very low immune-reaction, the CPC cells-F scaffold organoids induced CD3+ lymphocyte activation even in immunocompromised animals. This unexpected reaction suggests that it is not possible to create an *in vivo* model of a cardiac organoid using these constructs.

2. Materials And Methods

2.1. Material preparation

2.1.1. *Fibroin-water solution*

Bombyx mori cocoons (kindly provided by Socio Lario, Cassina Rizzardi, Como, Italy) were boiled for 1.5 hours in an aqueous solution containing 1.1 g/L Na₂CO₃ (10 g of silk/L of solution) and then for another

1.5 hours in a water bath containing 0.4 g/L Na₂CO₃. The cocoons were rinsed thoroughly with warm distilled water to extract the residual glue-like sericin proteins and then air-dried.

The fibroin-water solution was prepared by dissolving fibroin in an aqueous solution containing 9.3 M LiBr (10% w/v, Fluka Chemical, St. Louis, MO, USA) at 65°C for 2 h, followed by dialysis (3 days) against distilled water with a 3,500 Da M_w-CO membrane (Slyde-A-Lyzer, Pierce, Rockford, USA) to eliminate the salt. The resulting solution was concentrated by dialyzing against a Polyethylene Glycol (PEG)-water solution (25% w/v) for 5 hours and filtered through a 160–250-mm filter (Duran Group, Wertheim/Main, Germany). The final concentration of silk fibroin in the aqueous solution was approximately 15% w/v, as determined with a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) (A280).

2.1.2. Freeze-dried sponges

3D porous fibroin scaffolds were prepared using a freeze-drying technique with varied parameters to obtain different pore sizes and orientations. The aqueous fibroin solution was diluted to 5%, poured into polystyrene Petri dishes, frozen at -80°C, and freeze-dried (sample RP). Sponges with an oriented structure were also obtained using the 5% fibroin solution; however, the Petri dish was positioned vertically to induce a temperature gradient (sample O). All obtained samples were stabilized by treating with a methanol/water solution (80/20 v/v) for 10 min, rinsed several times with distilled water to eliminate the solvent, and freeze-dried again.

2.1.3. Electrospun nets

The aqueous fibroin solution was frozen at -20°C, lyophilized at -50°C, and stored in a desiccator until use. A 15% w/v solution was prepared dissolving the freeze-dried fibroin in formic acid (98–99%). A 3-ml syringe with a metal needle was filled with the solution and mounted in a programmable syringe pump (Model 11 Plus, Harvard Apparatus, Holliston, MA, USA) set at a constant flow rate of 0.002 mL/min. A positive voltage (15 kV or 20 kV) was applied to the solution by the needle, while a rotational mandrel, which was covered with aluminum, was connected to the ground electrode. The deposition was performed at room temperature (RT) at constant rotational speed (3,250 rpm) for 8 hours. The resulting non-woven nets were stabilized in methanol/ water (80/20 v/v) for 10 min and washed in distilled water for 2 days to remove the residual solvent (sample F).

2.2. Sample morphological evaluation by field-emission scanning electron microscopy (FE-SEM)

All samples were examined in a dry state with a field emission-scanning electron microscope (FE-SEM, Supra 40, Zeiss, Oberkochen, Germany) after coating with gold in a reduced argon atmosphere.

2.3. CPC isolation and culture

Adult Sprague-Dawley rats (up to 8-months old) were anesthetized with vaporized isoflurane, 0.20 mg/kg intramuscular (IM) Zoletin 20, 0.25 mg/kg IM medetomidine, and 0.0025 mg/kg SC atropine. The hearts were excised away from the chests while they were still beating. Each excised heart was placed directly

into a Falcon tube with 50 mL of Hank's balanced salt solution (HBSS; Invitrogen, Life Technologies Corp., Carlsbad, CA, USA) with 50 U/mL collagenase II (Life Technologies Corp.) and 3 mmol/L CaCl₂ to prevent blood coagulation and to allow for the rapid penetration of the collagenase solution directly into the coronary vessels. The atria were separated from the ventricles in a laminar flow hood. The ventricles were cut into four pieces, and these pieces (1 heart/tube) were placed into 20 mL fresh HBSS with 50 U/mL collagenase II and 3 mmol/L CaCl₂. The solution was collected following a 10-min incubation period at 37°C in a rotating dry incubator, and pieces were left on the bottom of the 50-ml Falcon tube. Isolated cells were removed from the collagenase solution by centrifugation and put into fresh M-199 medium (BD Biosciences, Franklin Lakes, NJ, USA) supplemented with 20% fetal bovine serum (FBS; Biolife Italiana S.r.l., Milano, Italy), 3 mg/mL fungizone, 300 mg/mL streptomycin, and 300 U/mL penicillin (antibiotic-antimycotic 100×, Cat. No. 15240-062, Invitrogen, Life Technologies Corp., Grand Island, NY, USA). Another 20 mL of HBSS with 50 U/mL collagenase II and 3 mmol/L CaCl₂ was added to the 50-ml Falcon tube with the four pieces, which was incubated for another 10 min at 37°C. We then performed repeated centrifugations as previously described [1, 8]. Only the second cell fraction (enriched in c-Kit⁺/Sca-1⁺ cells) was plated into 75-cm² poly-d-lysine-coated BD flasks with fresh M-199 medium supplemented with 20% FBS. The first fraction was discarded. After allowing the cells to grow, cells were mildly harvested with a solution of 0.05% porcine trypsin and 0.02% EDTA (trypsin-EDTA solution 10×, T4174, Sigma-Aldrich, St Louis, MO, USA) in phosphate buffered saline (PBS) or HBSS for no more than 2 min; cells that did not detach from the plasticware were cardiac fibroblasts and were discarded. To maintain an undifferentiated state, cells were cultured at 600,000 cells/25 cm² in M-199 supplemented with 20% FBS and harvested two days per week with a trypsin-EDTA solution (Sigma-Aldrich).

2.4. Organoid formation technique

Cells were cultured in a 50 µm-thick collagen I gel (rat tail collagen, diluted 1:8; BD Biosciences, Sparks, MD, USA) with or without scaffolds in the inserts in 24-well plates (2 × 10⁵ cells/insert; BD Biosciences). The previously used open pore polylactic acid (OPLA) scaffold (BD Biosciences), from here on referred to as sample BD, was considered a positive control for differentiation in 3D cultures [8]. Scaffolds with cells were placed in 300 µl diluted collagen, covered with M-199-20% FBS medium and incubated for 21 days in a cell culture incubator with medium changed twice a week.

2.4. Flow cytometry

Cells were detached from flasks, counted, and placed in FACS tubes (200,000 cells/sample). Cells were washed once with PBS and fixed with 4% paraformaldehyde in PBS. After washing, cells were blocked with incubation buffer (IB, PBS:M-199 supplemented with 10% FBS, 1:9 v:v), placed in the primary antibody solution in IB RT [anti-c-Kit, KAP-TK005, (Stressgen Bioreagents, Ann Arbor, MI, USA); diluted 1:200] for 45 min, rinsed twice in PBS, and then incubated in the secondary antibody solution (diluted 1:200 in IB for 45 min RT). After rinsing with PBS, cells were analyzed using a FACSVerse Flow Cytometer (BD Biosciences).

2.5. Animal models

CPC cells alone, scaffolds alone, or CPC cell-scaffold constructs were injected into 5-week-old athymic nude-*Foxn1^{nu}* (nude) mice, CB-17/IcrHan®Hsd-*Prkdc^{scid}* (SCID) mice, and athymic Hsd:RH-*Foxn1^{nu}* (nude) rats (Harlan Laboratories Inc., Indianapolis, IN, USA). All experimental protocols used in this study were approved by the Animal Care and Use Committee of the "Ministero della Sanità", experiments performed before the entry into force of Decree Law n. 26/2014, in application of European Directive 2010/63/Eu.

2.6. Organoid microinjection

Each type of scaffold or organoid was injected with a 0.5-mm trocar in the dorsal SC region of the nude mice, SCID mice, and nude rats, with one organoid per animal. The animals were kept in captivity for 45 days. After that period, they were sacrificed by displacement of the first cervical vertebra, and the bumps were excised, fixed in a solution of acetone:methanol:water (2:2:1 v/v) and dipped in paraffin. Finally, the embedded bumps were cut into 5- μ m sections and stained with hematoxylin and eosin (H&E) or Masson's trichrome staining.

2.7. Tumorigenicity assays

2.7.1. In vitro

A tumorigenic control of CPC cells was assessed *in vitro* and *in vivo*. CPC cells, VERO cells (negative control) and Hep-2 cells (positive control) were cultured *in vitro* in three different 75-cm² flasks (BD Biosciences) as described in Furesz et al [11]. Later, each cell culture (1×10^5 cells) was inoculated in 6-well plates with a solid medium consisting of MEM and Agar Noble (Sigma-Aldrich) and incubated at 37°C. Cells were observed under an inverted microscope for 3 weeks. The number of colony forming units (CFUs) was counted and compared with the control (Hep-2).

2.7.2. In vivo

CPC cells, VERO cells, and Hep-2 cells were inoculated *in vivo* by intracutaneous injection. Each cell type was injected into 10 nude mice. The animals were observed for 21 days and the formation of new nodules in the injection area was noted. The size and weight of the neoplasms were measured to evaluate their growth after 21 days. The experiment was considered valid if at least 9 mice inoculated with the positive control cells (Hep-2) produced neoplasms.

2.8. Histological analysis of scaffolds or constructs in vivo

2.8.1. H&E staining

Paraffin sections were stained with H&E and used to assess the presence of scaffolds in the biopsies, scaffold integration, and appearance of the constructs. Biopsies or scaffolds containing cells were fixed in an acetone:methanol:water solution (2:2:1) for 12 h, washed in tap water, and dehydrated with ethanol at 30, 50, 70, 95, and 100% v/v. After dehydration, the tissue pieces were placed in xylol for 1 h and embedded in paraffin. The paraffin-embedded tissue samples were cut into 5- μ m sections. The sections

were deparaffinized with xylene for 10 min and hydrated with a decreasing ethanol gradient. They were stained with hematoxylin (Merck KGaA, Darmstadt, Germany) for 4 min, blocked in tap water for 15 min, treated with eosin (Merck KGaA) for 1 min, and rinsed in water. Sections were dehydrated and mounted with Canada balsam (Panreac Química S.L.U., Barcelona, Spain). Images were taken with a Leica DM5000 microscope (Leica Microsystems, Wetzlar, Germany).

2.8.2. Analysis of fibrosis and giant cells using Masson's trichrome staining.

Paraffin-embedded sections were stained with Masson trichrome staining kit (Bio-Optica Milan, Italy) according to the manufacturer's instructions to assess the extent of fibrosis around the implants and the presence of giant cells. The collagen, nuclei, and cell cytoplasm appeared blue, black, and red, respectively. Images were taken with a Leica DM5000 microscope (Leica Microsystems).

2.9. Immunohistochemistry

Immunohistochemistry was performed with a biotin-free method using a specific probe to detect rabbit antibodies, followed by an HRP polymer that binds to both the probe and rabbit antibodies (MACH 1 Kit, Biocare Medical, Concord, CA, USA). After deparaffinization and rehydration, tissue sections were incubated with a 3% hydrogen peroxide solution (Sigma-Aldrich) for 5 min. After incubation with the protein blocking agent Background Sniper (Biocare Medical, Concord, CA, USA) for 15 min, the tissue sections were incubated with an anti-CD3 antibody (diluted 1:100, rabbit polyclonal, A0452, DAKO Denmark A/S, Glostrup, Denmark) for 1 hr. The linked primary antibody was detected using the HRP-streptavidin peroxidase system according to the manufacturer's instructions. Biocare's Betazoid DAB was used as the chromogen. The sections were then washed in tap water and counterstained with hematoxylin for 1 minute. VectaMount (Vector Laboratories Inc., Burlingame, CA, USA) was used as mounting medium.

2.10. Confocal scanning analysis

Cells cultured on poly-D-lysine(Sigma-Aldrich)-coated chamber slides were first fixed with 4% paraformaldehyde for 30 min and then with ice-cold methanol for 30 min. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0) containing 0.05% Tween 20 for 10 min. After 30 min incubation with 5% bovine serum albumin (Sigma-Aldrich), cells were incubated overnight at 4°C with primary antibodies [dilution 1:100; anti-CD11b (integrin α M), clone M-19, sc-6614, Santa Cruz Biotechnology; anti-IL -4, clone M-19, sc-1261, Santa Cruz Biotechnology; anti-IL -13, clone M-17, sc-1776, Santa Cruz Biotechnology]. The linked primary antibodies were detected with a FITC-conjugated secondary anti-goat antibody (Sigma-Aldrich) at a dilution of 1:50. Nuclei were stained with 1 μ M TOTO - 3 stain in PBS (T3604, Life Technologies) or Hoescht staining for 5 min. Images were captured using a ZEISS LSM710 Exciter Laser Scanning Confocal Microscope.

2.11. Fluorescence in situ hybridization (FISH)

Paraffin-embedded sections were kept at 37°C overnight and then at 60°C for 10 min. The sections were deparaffinized in xylol at 60°C for 10 min, hydrated through a graded ethanol series, and immersed in citrate buffer containing 1% Tween 20 for 15 min for heat-induced target retrieval. Slides were cooled for 5 min at RT and placed in PBS containing 0.1% Tween (Sigma-Aldrich). To catalyze RNA degradation, slides were treated with 10 µg/µL RNase at 37°C for 1 hr. FISH was performed on the sections by hybridization with a custom-made N-terminal Cy3-labeled peptide nucleic acid (PNA) probe that recognizes mouse centromeres but not rat centromeres (N-ATTTCGTTGGAAACGGGA-C, Exiqon, Vedbaek, Denmark). The sequence was adopted from Vander Griend et al [12]. The PNA probe was used at a concentration of 6.4 pmol in hybridization buffer (50% formamide, 2× SSC, pH 7), and the sections were denatured at 75°C for 5 min, followed by hybridization at 60°C for one hour. Sections were washed three times with 0.1× SSC at the hybridization temperature for 5 min and twice with 4× SSC containing 0.05% Tween 20 at 37°C, followed by washing in PBS at RT for 5 min. Nuclei were counterstained with a 1:1000 dilution of TOTO – 3 (Life Technologies) in PBS at RT. Sections were mounted with Vectashield (DAKO) for confocal analysis.

3. Results

3.1 Scaffold characterization

The morphological features of the silk fibroin constructs were observed using FE -SEM, as shown in Fig. 1.

The 3D porous structure obtained from the freeze-dried fibroin-water solution exhibited an interconnected homogeneous pore distribution (Fig. 1a). The sponges, on the other hand, exhibited a very distinct bimodal pore size distribution, which was achieved by inducing a temperature gradient (Fig. 1b). The fibers of the electrospun net were randomly distributed with a homogeneous fiber diameter size, as shown in Fig. 1c.

3.2 Cell isolation, characterization and organoid formation

We isolated immature cells from the heart of mature female rats using a differential adhesion method as previously described [8–9] (Fig. 2b). Using poly-D-lysine and mild digestion with trypsin-EDTA solution, we obtained an adequate amount of highly proliferative cells two weeks after tissue digestion. The isolated cells were then characterized by FACS analysis and were highly positive for c-Kit compared to the isotype control, as previously described [1, 9] (Fig. 2a). Even after multiple passages in culture, cryopreservation, and thawing, these cells were able to express the stem cell markers c-Kit, Sca-1, and MDR-1 (Fig. 2e-f-g), as well as Titin, a structural cardiac protein of the sarcomere (Fig. 2d).

Two weeks after tissue digestion, cells were tested for tumorigenicity *in vitro* and *in vivo*. 7–10 days after the start of the *in vitro* tumorigenicity assay, the tumorigenic cells (Hep-2) started to proliferate and form multicellular aggregates, whereas the negative cells (VERO) and CPC cells showed atrophy. Therefore, the tumorigenicity assay performed *in vitro* on the isolated CPC cells was negative (data not shown).

The tumorigenicity assay *in vivo* was positive for the Hep-2 cells (positive control) and negative for the VERO (negative control) and CPC cells. Solid tumors appeared in all nude mice injected with Hep-2 cells, whereas there was no evidence of tumors in the various organs analyzed from nude mice injected with VERO and CPC cells (Supplemental Fig. 1).

Having established that CPC cells are non-tumorigenic, we prepared and tested CPC-derived organoids for injection into immunocompromised animals. C-kit-positive cells were cultured *in vitro* in a collagen I gel for 21 days in a BD insert, as shown in Fig. 3A-B. The CPC cells formed a compact mass, well organized, with newly developed small vessels (Fig. 3c-d, as previously described [8]) and able to increase the expression of structural cardiac markers such as cardiac Troponin T2 (Fig. 3e-f-g).

3.3. Testing CPC-collagen Organoids *in vivo*

To understand whether cardiac organoids composed of CPC cells embedded in collagen I and grown in silk fibroin scaffolds, as previously described (Silk Fibroin paper), can be used to create a vascularized and functional organoid *in vivo*, we first tested the viability and differentiation of CPC cell-collagen organoids *in vivo*, then the host response to biomaterials, and then the host response to the CPC cell-scaffold-collagen combination in immunosuppressed animals.

A collagen gel was used to implant the cells and minimize cell dispersion. A collagen I gel was chosen as the embedding medium because we have previously shown that it is a good substrate to induce cardiac differentiation of rat CPC cells *in vitro* within 21 days [8]. A group of mice transplanted only with a collagen I gel without cells was included as a control; the gel alone and surgical manipulation did not induce an inflammatory response or attract immune cells to the engraftment site.

We injected CPC cell collagen organoids (Cell + coll) into the SC region of nude mice at time 0 (Fig. 4o-q). Samples were evaluated at two time points, 21 and 45 days, and the results were the same. Twenty-one days was the incubation time required for CPC cells to differentiate to the cardiac phenotype *in vitro* [1, 8] and the time required to rule out the appearance of solid tumors. Forty-five days was also examined to allow complete differentiation of CPC cells.

The organoids grew and the cells proliferated, but at the same time induced a foreign body reaction, with nodule formation and an infiltrate of lymphocytes, as shown by both H&E staining in Fig. 4o and 4q and immunohistochemistry for CD3 + positive lymphocytes in Fig. 5d.

Collagen remnants were still visible in the center of the encapsulated area (Fig. 4o, clear zone). The massive presence of lymphocytes was highlighted by Masson's trichrome staining (Fig. 4p).

Furthermore, using a FISH for mouse centromeres, we demonstrated that the implanted rat CPC cells were undetectable in SCID mice after 21 and 45 days (Fig. 5e). Anatomopathological evaluation of all organs showed that they did not induce tumor formation.

These experiments suggest that the cells of rats were destroyed by the unfavorable immune response. As shown in Fig. 5e, the vessels present in the biopsies were also of murine origin. The same infiltrate was present in nude rats when rat CPC cells were implanted (Supplemental Fig. 2c).

3.4. Host reaction to biomaterials

Before investigating the fate of CPC cell silk fibroin organoids *in vivo*, we examined the host response to the biomaterials alone in nude mice, SCID mice, and nude rats. Skin biopsies were collected 45 days post-injection, fixed and embedded in paraffin for further analysis. The implanted BD (as a control), silk fibroin sponges, and electrospun nets were readily identifiable in the H&E-stained paraffin-embedded sections because of the size of the scaffolds, their morphology, and the intense staining of the tendons forming them (Fig. 4).

Implanted BD, silk fibroin scaffolds and meshes were examined *in vivo* after 45 days of incubation, and they did not appear to have undergone significant biodegradation (Fig. 4a, d, g, l). Fragments of BD, RP, and O scaffolds were encapsulated (Fig. 4a, d, g). The voids encompassed by the chords of the implanted scaffolds were filled with dermal fibroblasts, and the chords were covered with macrophages and giant cells (Gc - Fig. 4b-c, f-g, i). An amorphous extracellular matrix (ECM), cells of different types, thin bundles of collagen fibrils filled the interstitial spaces (Fig. 4b, e, h). No clusters or infiltrates of lymphocytes and/or plasma cells were detected. Giant cells were particularly evident in sections stained with both Masson's trichrome and H&E (Fig. 4b-c, f, i). The appearance of F (Fig. 4l-n) was different from the porous scaffolds (Fig. 4d-i) and so was the induced host response. At 45 days post-injection, there was hardly any capsule left (Fig. 4l), and no macrophages were seen (Fig. 4m-n). Fibroblasts populated the surface of the F net, and no lymphocytes were present (Fig. 4m-n).

To confirm the presence of giant cells and the induction of a foreign body reaction, we assessed the expression levels of CD11b, IL -4, and IL -13 by immunofluorescence and confocal microscopy analysis. CD11b, a surface marker of giant cells [13], was expressed on the surface of macrophages attached to the tendons of the control scaffold BD, silk fibroin scaffolds RP and O (Fig. 3).

IL -4 and IL -13, two interleukins expressed during foreign body reaction that induce macrophage fusion on the surface of scaffolds [13–14], were detected in BD, RP and O samples (Fig. 3). In samples of skin biopsies with silk fibroin F nets CD11b, IL -4 and IL -13 were barely expressed.

3.5. Fate of CPC cells-F scaffolds

Because the F fibers were the least reactive, we implanted them along with the CPC and collagen (Sc + cell) in the subcutaneous area of nude mice. However, even though the F-scaffolds alone did not induce a foreign body reaction with giant cells, the CPC cells were probably destroyed by the lymphocyte infiltrate, as shown by the FISH analysis in Fig. 3f.

4. Discussion

In the present study, we investigated the possibility of using CPC cells, collagen, fibroin-based substrates, two 3D porous matrices, and an electrospun net, to create a vascularized and functional *in vivo* model of a cardiac organoid. We tested first the viability and differentiation of CPC cell-collagen organoids *in vivo*, second the host response to biomaterials, and then the host response to the CPC cell-scaffold-collagen combination in immunocompromised animals.

Scaffolds with three different geometries were prepared using a fibroin-water solution, including two sponges with different pore sizes and pore distributions and an electrospun net with randomly distributed fibers. Characterization of the morphological properties of the fibroin-based constructs showed that the pore size and pore distribution of the sponges were tuned according to the different protocols used to fabricate the scaffolds, which included freeze-drying and a temperature gradient in the quenching step. The results of the morphological analysis showed a homogeneous pore distribution and bipolar pore size with a well-oriented lamellar structure for the sponges. The electrospun net was found to contain randomly distributed fibers with a constant diameter. The pore size ranges and water absorption capacity of the scaffolds were previously measured and published [15–17].

All porous scaffolds induced a foreign body reaction with giant cells and capsule formation. The electrospun net (F-scaffold) did not induce an evident foreign body reaction, but a small capsule was still visible. The foreign body reaction was confirmed by the presence of CD11b + giant cells and the expression of the inflammatory interleukins, IL -4 and IL -13.

Silk fibroin can be isolated in sufficient quantities from the cocoons of the silkworm *Bombyx mori* and is considered an adjustable and versatile commercially available biomaterial. The biocompatibility of silk fibroin can be improved by separating the immunogenic protein family, sericins, as we did in the present study, so that this biomaterial elicited only a moderate inflammatory response during subsequent implantation in rodents [18]. The biocompatibility of scaffolds has been shown to depend on a number of different parameters, including microstructure and architecture, both of which influence the inflammatory response. In addition, implantation of hybrid constructs, i.e. scaffolds pre-seeded with cells, can trigger an adaptive immune response towards the biological component and thus influence the host response to the implanted device [19].

The CPC cells that we were able to isolate as primary cultures from adult rat hearts after a long collagenase digestion [8] did not induce tumor masses in the several organs analyzed, nor did they induce CFUs *in vitro*. We verified that the cells expressed the usual stem cell markers (c-Kit, Sca-1 and MDR-1) and some structural proteins typical of the heart in both 2D and 3D cultures (Titin and cardiac Troponin T2), as studied by Bernstein and Srivastava [20]. In fact, we had already shown in a previous work that c-Kit + CPC cells isolated by repeated treatments with collagenase and cultured in 3D under different conditions can differentiate and express many cardiac proteins *in vivo* [8–9]. However, even though several research groups have isolated similar cardiac progenitor cells, no one has yet investigated their applicability as the main component of an *in vivo* model of a cardiac organoid. Because their immunogenicity has not been studied *in vivo*, we also examined the immune response induced by

injection of the CPC cell-collagen organoid, the host response to the CPC cell-scaffold-collagen combination in immunosuppressed animals.

Unfortunately, injection of the CPC cell-collagen organoids induced a cell-mediated immune response with the formation of a capsule. The implanted cells were completely destroyed by the immune reaction.

Because the F-fibers were the least reactive scaffolds studied, we implanted them together with the CPC cells and collagen (Sc + cell) into the subcutaneous area of nude mice, but even though the F-scaffolds alone did not induce a foreign body reaction with giant cells, the CPC cells were destroyed by the lymphocyte infiltrate.

A foreign body reaction usually occurs when biomaterials alone are implanted for therapeutic clinical applications in tissue engineering. This reaction is the starting point for tissue regeneration in orthopedics because the foreign body reaction stimulates the regeneration of germinal/progenitor cells of the host tissue while degrading the scaffolds in a reasonable time frame [21]. However, this phenomenon is not useful for stem cell therapy or *in vivo* modeling of vascular and functional organoids because, as shown here, the foreign body response destroys the cells very quickly after implantation, even when injected into immunosuppressed animals.

Organoids are structures that arise from the cells of a particular organ, or from a combination of different, partially differentiated tissues, or from progenitor cells of the organ itself that are capable of giving rise to the various tissues of the organ. They have gained importance in scientific research because they represent a valid alternative to animal experiments, and because they can be a valid tool for testing drugs, new molecules or even simulating the environment of a particular pathology. The use of organoids reduces the number of animals used for experiments.

Many organoids arise from mesodermal cells and are formed from a single mass of progenitor cells. When cells in the center of the mass are deprived of nutrients and oxygen, necrosis occurs, even *in vitro*. Our progenitor cells (CPC cells) are naturally capable of giving rise to cells of endothelial origin, as we have shown previously [8], and are capable of forming vessels even *in vitro* within the collagen I gel. For this reason, we hoped that the same mass of cells could develop an adequate network of vessels in a more complex environment such as the subcutaneous region of immunocompromised mice. Vascular organoids are important both to ensure the supply of nutrients and oxygen to all cells in the mass and to provide a mesenchymal niche useful for maintaining organoid structure [22]. Previous studies have shown that induced human pluripotent stem cells (hPSCs) are able to differentiate into a microvascular network and this was integrated with the host vasculature to form a functional blood system when implanted into immunodeficient mice [23].

Here, we used FISH to show that the vasculature present in the implanted organoids was derived from the host and not from the donor cells.

In conclusion, CPC cells are capable of expressing cardiac structural markers and organizing a vascular network in vitro, but when implanted (with or without fibroin nets) into the subcutaneous region of immunocompromised mice, they are likely to be destroyed by CD3 + lymphocytes, which are still active in this type of animals.

Declarations

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Conflicts of interest/Competing interests No author has a conflict of interest.

Ethics approval This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Italian Ministry of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Istituto Zooprofilattico Sperimentale Sicilia. All efforts were made to minimize suffering. Experiments conducted before the entry into force of the Decree Law n. 26/2014, in application of the European Directive 2010/63/Eu.

Consent to participate All authors agreed to participate in the study

Consent for publication All the authors viewed the manuscript before it was sent and gave their consent

Availability of data and material The original data is available upon reasonable request

Code availability Not applicable

Authors' contributions M.A, M.C. provided silk and fibroin scaffolds; M.F., G.A., P.D.M., C.G. provided animals and performed experiments on animals; B. R.,R.P. performed Confocal Analysis; G.F., P.F. isolated cells from rat hearts and created organoids in vitro; D.F.V. paper writing, data analysis, light microscopy analysis.

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Figures

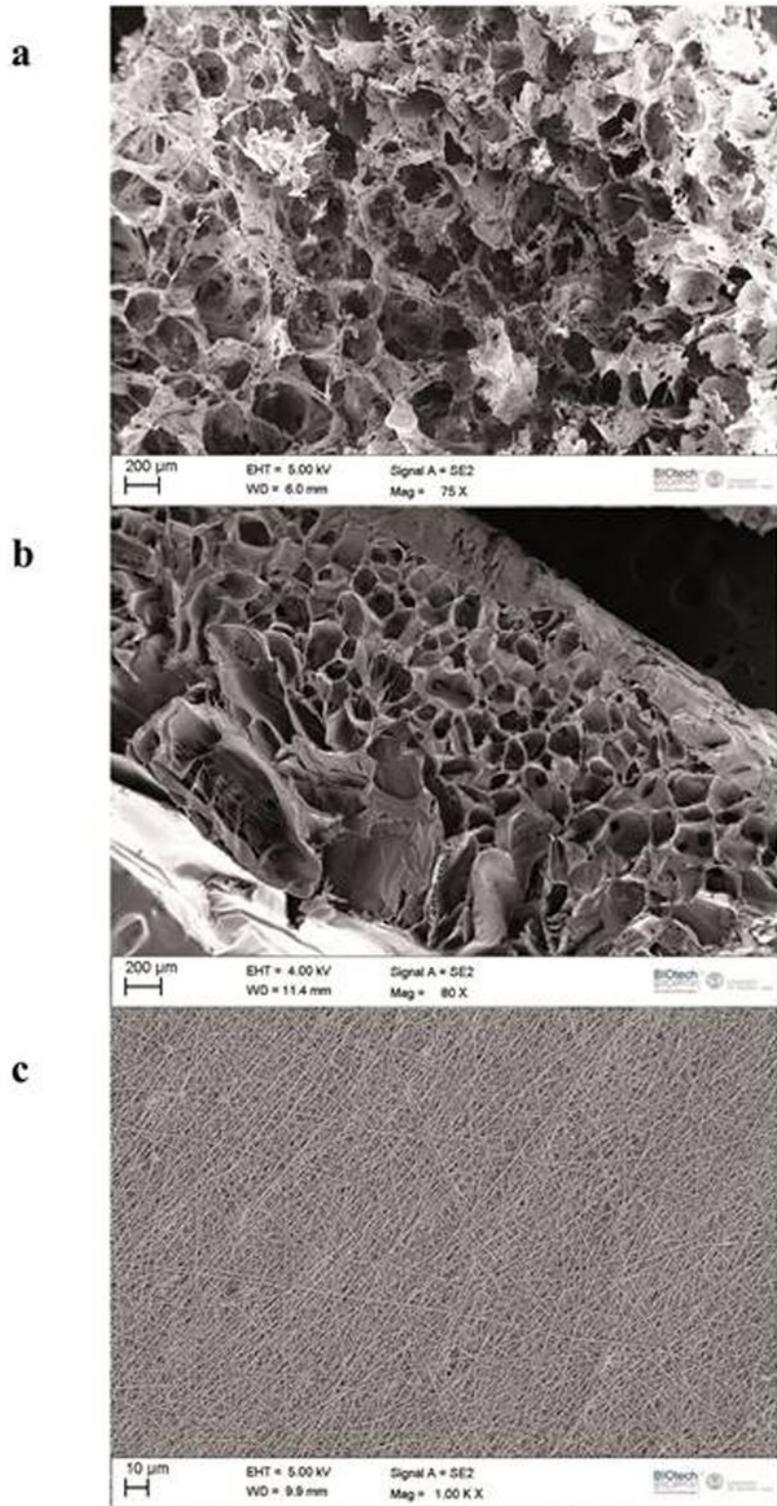


Figure 1

Field Emission-Scanning Electron Microscope (FE-SEM) micrographs of silk fibroin constructs. Freeze-dried sponges with homogeneous pore distribution, sample RP (a); sponges with bimodal pore distribution, sample O (b); electrospun net, sample F (c).

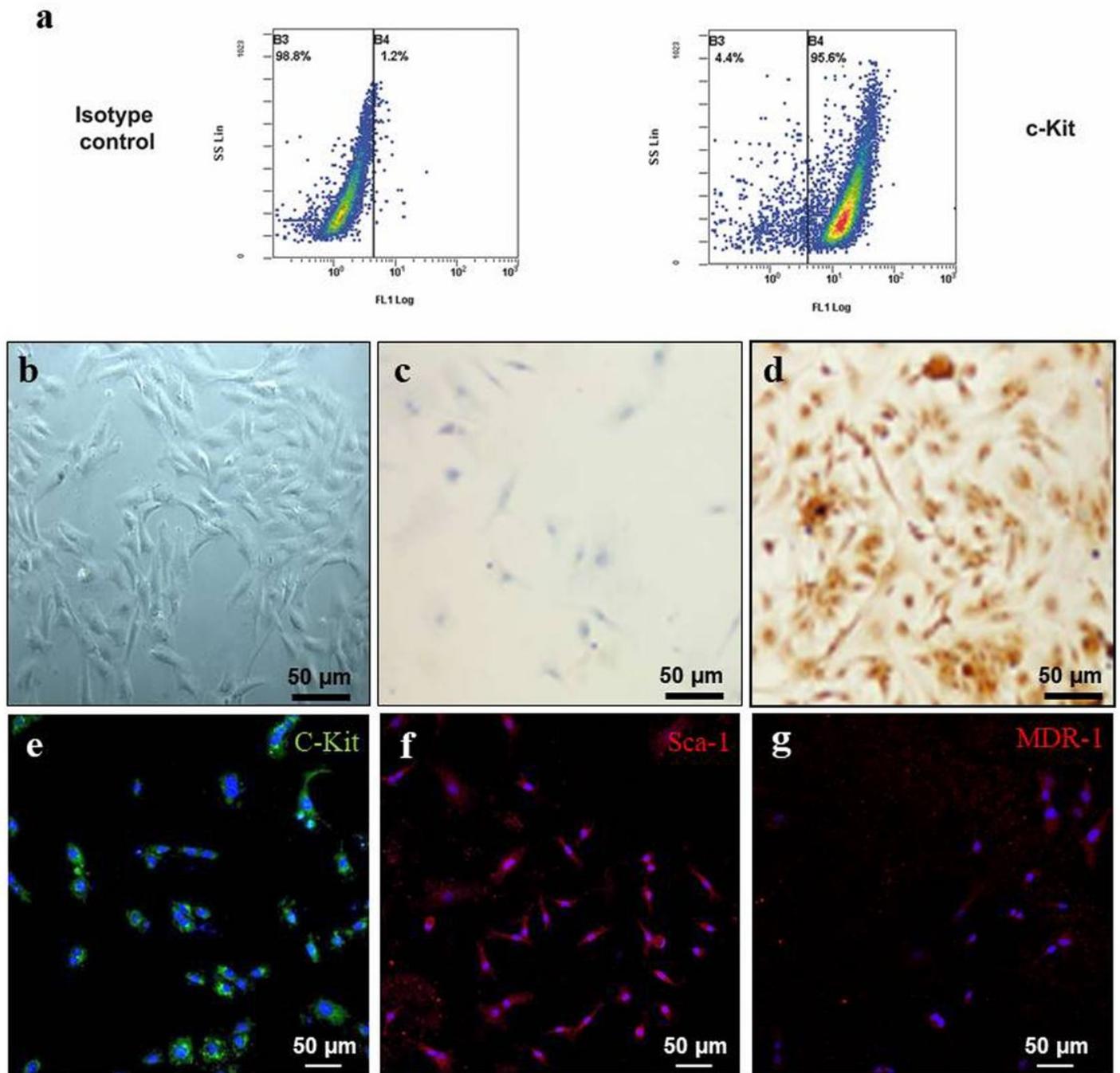


Figure 2

A - FACS analysis of isolated CPC cells showing the percentage of cells expressing c-Kit ten days after tissue digestion. a, non-immune isotope antibody was used as negative control; b, CPC cells in 2D culture; c, negative control of d; d, Titin expression in CPC cells in 2D cultures; e-f-g c-Kit, Sca-1, and MDR-1 expression each analyzed by Confocal microscopy.

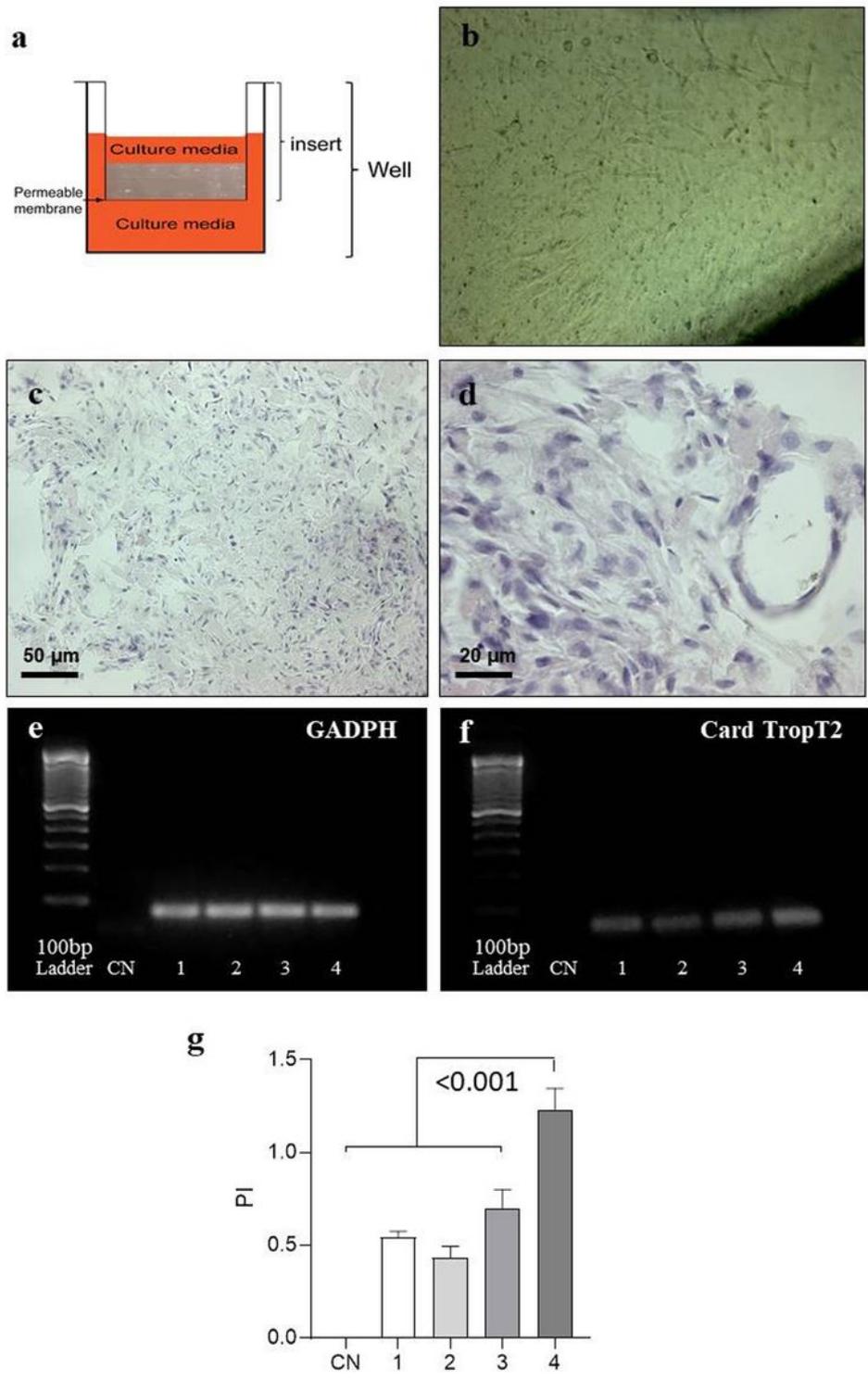


Figure 3

A - Diagram showing the CPC cells in 3D culture; b - CPC cells cultured on the insert; c - d paraffin embedded sections of a CPC cell collagen organoid in vitro stained with H&E; e - GADPH expression in CPC cell collagen organoids; f - Cardiac Troponin T2 expression in CPC cell collagen organoids; g - Cardiac Troponin T2 expression normalized with GADPH. CN: negative control; 1: CPC grown in flasks; 2:

CPC grown in collagen for 12 days; 3: CPC grown in collagen for 16 days; 4: CPC grown in collagen for 21 days.

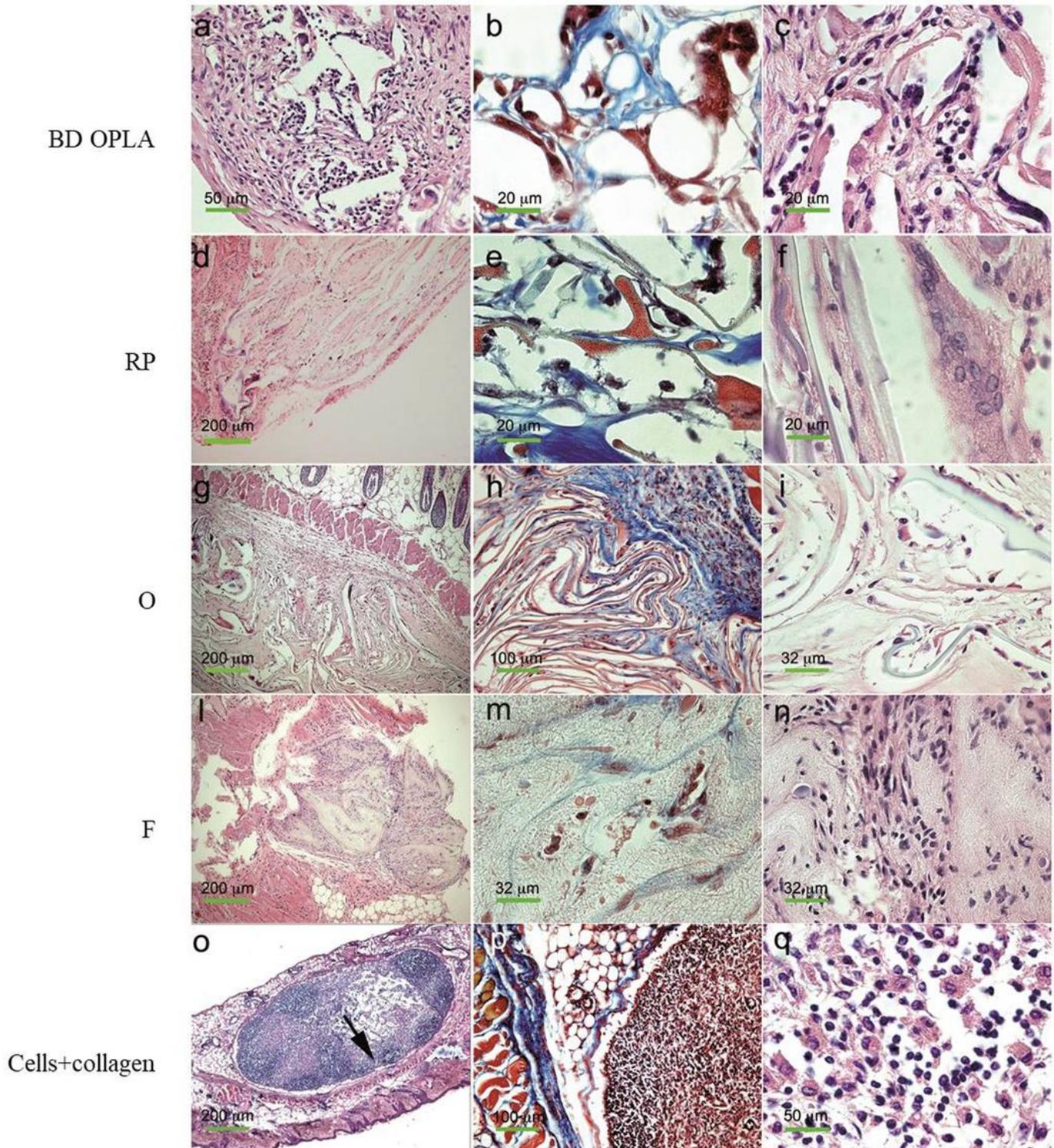


Figure 4

Morphological analysis of in vivo reaction to biomaterials and constructs. Small pieces (2 mm²) of biomaterials alone were implanted subcutaneously into nude mice to evaluate immune response. After 45 days, biopsies were harvested, embedded in paraffin, and sections stained for further analysis. H&E

staining of BD (a, c), RP (d, f), O (g, i), F (l, n), and CPC and collagen I constructs (o, q); Masson's trichrome staining of BD (b), RP (e), O (h), F (m), and CPC and collagen I constructs (p). Sc = scaffold; ECM = extracellular matrix; Lymph = lymphocytes; Gc = Giant cells; Capsule = fibrous capsule of the foreign reaction; Gaps = holes left by the implanted scaffold; Muscle = muscle of the skin.

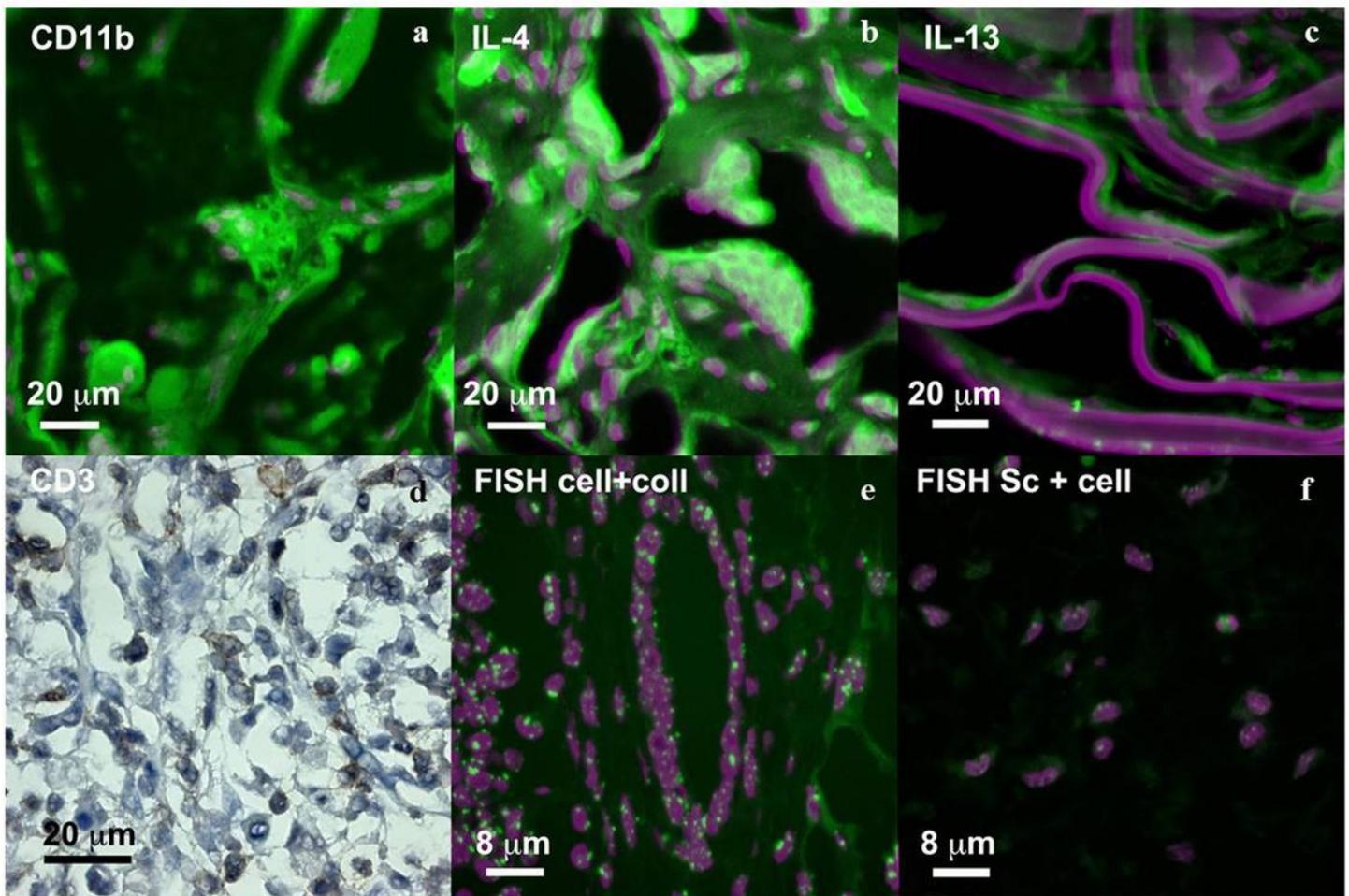


Figure 5

Foreign body reaction to biomaterials. BD , RP-, O-, and F-scaffolds induced a IL -4/ IL -13-mediated foreign body reaction with CD11b+ giant cells similar to that shown in a, b, and c, respectively. Nuclei were stained with Toto-3 (purple), and scaffolds also reacted with Toto-3, which appears purple. d - Infiltrate of CD3+ lymphocytes in CPC cell collagen organoids implanted in the SC of nude mice. e - FISH showing only mouse-positive cells in biopsy of CPC cell-collagen organoids implanted in nude mice. f - FISH showing only mouse-positive cells in biopsy of CPC cell-collagen F scaffold.

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