



Consequences of ineffective decellularization of biologic scaffolds on the host response

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ABSTRACT

Biologic scaffold materials composed of extracellular matrix (ECM) are routinely used for a variety of clinical applications. Despite known variations in tissue remodeling outcomes, quantitative criteria by which decellularization can be assessed were only recently described and as a result, the amount of retained cellular material varies widely among commercial products. The objective of this study was to evaluate the consequences of ineffective decellularization on the host response. Three different methods of decellularization were used to decellularize porcine small intestinal ECM (SIS-ECM). The amount of cell remnants was quantified by the amount and fragmentation of DNA within the scaffold materials. The M1/M2 phenotypic polarization profile of macrophages, activated in response to these ECM scaffolds, was assessed *in vitro* and *in vivo* using a rodent model of body wall repair. The results show that, *in vitro*, more aggressive decellularization is associated with a shift in macrophage phenotype predominance from M1 to M2. While this shift was not quantitatively apparent *in vivo*, notable differences were found in the distribution of M1 vs. M2 macrophages within the various scaffolds. A clear association between macrophage phenotype and remodeling outcome exists and effective decellularization remains an important component in the processing of ECM-based scaffolds.

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1. Introduction

The use of biologic scaffolds derived from decellularized mammalian tissues is commonplace. Such scaffolds are composed of extracellular matrix (ECM) and have been used to repair or replace a variety of damaged or diseased tissues including cardiac [1–3], esophageal [4,5], dermal [6], and musculoskeletal [7–11], among others. These materials are typically regulated as devices and marketed as surgical mesh products; however, these ECM-based scaffolds can also serve as an inductive template for tissue repair and regeneration [12–14]. Numerous commercial products composed of allogeneic or xenogeneic ECM are now available for clinical use (Table 1).

Results of preclinical and clinical studies with biologic scaffolds have varied from very successful [15–19] to complete failure [20–23]. The host response to these materials can be attributed to

factors such as the source species (e.g., human, porcine, equine, or bovine), the tissue from which the ECM is isolated (e.g., dermis, small intestine, or pericardium), mechanical loading [24,25], and the niche factors to which the scaffold is exposed following implantation. The decellularization, disinfection, and sterilization methods used during the manufacturing process can markedly influence the tissue remodeling response and functional outcome [26,27]. Despite these known variations in functional outcome, no quantitative criteria by which decellularization can be assessed have been suggested until recently [26] and as a result, the amount of retained cellular material varies widely among commercial products composed of decellularized tissues [28]. The consequences of ineffective or incomplete decellularization upon the host response have not been systematically investigated.

The innate and acquired immune response to non-autologous cells is well established and understood by the tissue and organ transplantation community. However, the response to acellular xenogeneic or allogeneic biologic scaffold materials is less well understood. The macrophage represents a key component of the host response. Macrophages are activated in response to tissue damage, infection, or the presence of foreign antigens and subsequently release a variety of cytokines and chemokines [29].

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Table 1

Source material and form of commercial ECM-based products available for therapeutic applications.

Product	Company	Material	Form
AlloDerm®	LifeCell™	Human skin	Natural
Axis™ dermis	Mentor	Human dermis	Natural
Bard Dermal Allograft	C R Bard	Cadaveric human dermis	Natural
CuffPatch™	Biomet Sports Medicine	Porcine small intestinal submucosa (SIS)	Cross-linked
DuraADAPT™	Pegasus Biologicals	Horse pericardium	Cross-linked
Dura-Guard®	Synovis Surgical	Bovine pericardium	Cross-linked
Durasis®	Cook® Medical	Porcine small intestinal submucosa (SIS)	Natural
Durepair®	TEI Biosciences/Medtronic	Fetal bovine skin	Natural
FasLata®	C R Bard	Cadaveric fascia lata	Natural
Graft Jacket®	Wright Medical Tech	Human skin	Natural
Oasis®	Cook® Biotech/Healthpoint	Porcine small intestinal submucosa (SIS)	Natural
OrthADAPT™	Pegasus Biologicals	Horse pericardium	Cross-linked
Pelvicol™	C R Bard	Porcine dermis	Cross-linked
Peri-Guard®	Synovis® Surgical Innovations	Bovine pericardium	Cross-linked
Permacol™	Covidien	Porcine skin	Cross-linked
PriMatrix™	TEI Biosciences	Fetal bovine skin	Natural
Restore®	DePuy	Porcine small intestinal submucosa (SIS)	Natural
SurgiMend®	TEI Biosciences	Fetal bovine skin	Natural
Surgisis®	Cook® Medical	Porcine small intestinal submucosa (SIS)	Natural
Suspend™	Mentor	Human fascia lata	Natural
TissueMend®	TEI Biosciences	Fetal bovine skin	Natural
Veritas®	Synovis® Surgical Innovations	Bovine pericardium	Cross-linked
Xenform®	TEI Biosciences/Boston Scientific	Fetal bovine skin	Natural

Macrophages are now recognized to assume a variety of phenotypes characterized by distinct functional properties, surface markers, and their secreted cytokine profile [30]. Polarized macrophages are referred to as either M1 or M2 cells, mimicking the Th1/Th2 nomenclature. Classically activated, M1 proinflammatory macrophages express IL-12high, IL-23high, IL-10low; metabolize arginine; produce high levels of inducible nitric oxide synthetase (iNOS); secrete toxic reactive oxygen and nitric oxygen intermediates and inflammatory cytokines such as IL-1 β , IL-6, and TNF. M1 macrophages are inducer and effector cells in Th1 type inflammatory responses. In contrast, M2, alternatively activated macrophages are induced by exposure to a variety of signals including the cytokines IL-4, IL-13, and IL-10, immune complexes, and glucocorticoid or secosteroid (vitamin D3) hormones. M2 activated macrophages express IL-12low, IL-23low, and IL-10high; have high levels of scavenger, mannose, and galactose receptors; produce arginase in the place of arginine which results in the secretion of ornithine and polyamines; are involved in polarized Th2 reactions; and possess the ability to facilitate tissue repair and constructive remodeling [29,31,32].

Previous studies have demonstrated the importance of macrophages in tissue repair and regeneration, particularly in applications involving biologic scaffolds [33–37]. Macrophages are responsible, in part, for the degradation of ECM scaffolds. Depletion of circulating macrophages severely attenuates scaffold degradation and the associated constructive remodeling response [37]. Non-crosslinked biologic scaffolds promote the expression of an M2 macrophage phenotype at the site of remodeling although a population of macrophages expressing markers of an M1 phenotype persists [34,36]. Tidball has demonstrated that it is the switch from an M1 to M2 phenotype that stimulates progenitor cell differentiation and constructive tissue remodeling while an early M1 response stimulates progenitor cell recruitment and proliferation [33]. The ability of biologic scaffolds to promote expression of an M2 macrophage phenotype may therefore be critical to their ability to promote constructive tissue remodeling. However, the mechanism by which these biologic scaffolds promote M2 macrophage expression is currently unknown.

It is known that cell death incites a series of events that typically results in the classic cascade of inflammatory processes including

polymorphonuclear leukocyte (PMN) and mononuclear cell accumulation, edema, fibroblast infiltration, and eventual scar tissue formation [38–40]. The presence of intact cells within a biologic scaffold material has been shown to elicit a greater proinflammatory response than use of the acellular biologic scaffold alone [36]. It is logical, therefore, that cell remnants within a partially decellularized tissue could elicit a proinflammatory response that would adversely affect a constructive tissue remodeling outcome. In fact, it has been shown that the presence of intact cells within implanted scaffolds can be associated with adverse remodeling [36]. In contrast, thoroughly decellularized biologic ECM scaffolds are known to promote a host response that is polarized towards the M2 macrophage phenotype and is associated with constructive tissue remodeling [34,36,37]. These two responses sit at the polar extremes of the host response to an implanted biologic scaffold. Little is known about which cell components stimulate an M1 macrophage response or if a threshold level for cellular material exists below which the M2 macrophage phenotype predominates. It has been suggested that the presence of mitochondria or mitochondrial DNA may be a stimulator of M1 macrophages given their primitive bacterial origin [41]. In light of the heterogeneity with regard to the amount and efficacy of decellularization in commercially available ECM scaffolds [28], a more thorough understanding of the effects of cell remnants upon the host response is needed.

The objective of the present study was to evaluate the effect of cell remnants (i.e., ineffective tissue decellularization) within biologic scaffolds upon *in vitro* and *in vivo* outcome measures. Three different methods were used to decellularize porcine small intestinal submucosal ECM (SIS-ECM). The amount of cellular material remaining was quantified by the amount and fragmentation of DNA. The polarization profile of activated macrophages in response to these ECM scaffolds was then assessed *in vitro* and *in vivo* using a rodent model of body wall repair.

2. Materials and methods

2.1. Harvest and preparation of ECM from porcine small intestine

Preparation of small intestinal submucosa (SIS) ECM has been previously described [42,43]. Briefly, jejunum was harvested from market weight

(240–260 lbs.) pigs and split longitudinally. The superficial layers of the tunica mucosa were mechanically removed. Likewise, the tunica serosa and tunica muscularis externa were mechanically removed, leaving the tunica submucosa and basilar portions of the tunica mucosa. To produce ECM with differing amounts of remnant cellular material, three different chemical treatments were performed on the tissue. In rank order of most to least decellularization, the SIS material was treated with: (1) 0.1% peracetic acid (PAA) for 2 h with mechanical agitation and subsequent 15 min washes with phosphate buffered saline (PBS), deionized water, PBS, and deionized water; (2) As above, but with the PAA treatment limited to 1 hr; or (3) PBS for 2 h followed by subsequent 15 min washes with PBS, deionized water, PBS, and deionized water. For *in vivo* studies the prepared ECM was vacuum pressed to form a 4-layer laminate. *In vitro* assays were performed on single layer sheets of SIS-ECM. All devices were lyophilized and sterilized using ethylene oxide.

2.2. Histologic assessment of decellularization

Representative samples of SIS with each amount of decellularization were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections were cut at 5 μ m thickness and stained with hematoxylin and eosin (H&E) and 4', 6-diamidino-2-phenylindole (DAPI) to identify the presence of any residual intact nuclei within the tissue samples.

2.3. Quantification of DNA content

DNA was extracted from representative samples of SIS prepared by each of the three decellularization protocols. One hundred mg of lyophilized, powdered SIS-

ECM was digested with proteinase K digestion buffer [100 mM NaCl, 10 mM Tris-HCl (pH = 8), 25 mM EDTA (pH = 8), 0.5% SDS, 0.1 mg/mL proteinase K] at 50 °C for 24 h. The digest was extracted twice using 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol. DNA was precipitated from the aqueous phase at –20 °C with the addition of 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH = 5.2). The DNA was then centrifuged at 10,000 g for 10 min and resuspended in 1 mL of TE buffer [10 mM Tris (pH = 8), 1 mM EDTA].

The concentration of each extracted DNA sample was determined using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad CA, #P7589) using the manufacturers recommended protocol. A standard curve was constructed by preparing samples of known DNA concentrations from 0 to 1000 ng/mL. Samples were read using SpectraMax M2 Plate Reader (Molecular Devices, Sunnyvale, CA). DNA samples were diluted to ensure their absorbencies that fell into the linear region of the standard curve.

To determine DNA fragment size, equal concentrations of extracted DNA from each sample were separated on a 2% agarose gel containing 0.5% ethidium bromide and visualized with ultraviolet transillumination using a reference 100 bp ladder (New England Biolabs, Ipswich, MA). All assays were performed in triplicate. A quantitative comparison of the specimens prepared by the three different decellularization methods was then completed using recently established guidelines [26].

2.4. *In vitro* culture of macrophages on SIS-ECM devices

Characterization of the macrophage response to devices with varying amounts of cell remnants was performed by *in-vitro* culture of mouse monocyte macrophage cells (RAW 264.7 from ATCC) on single layer sheets of SIS-ECM prepared using the

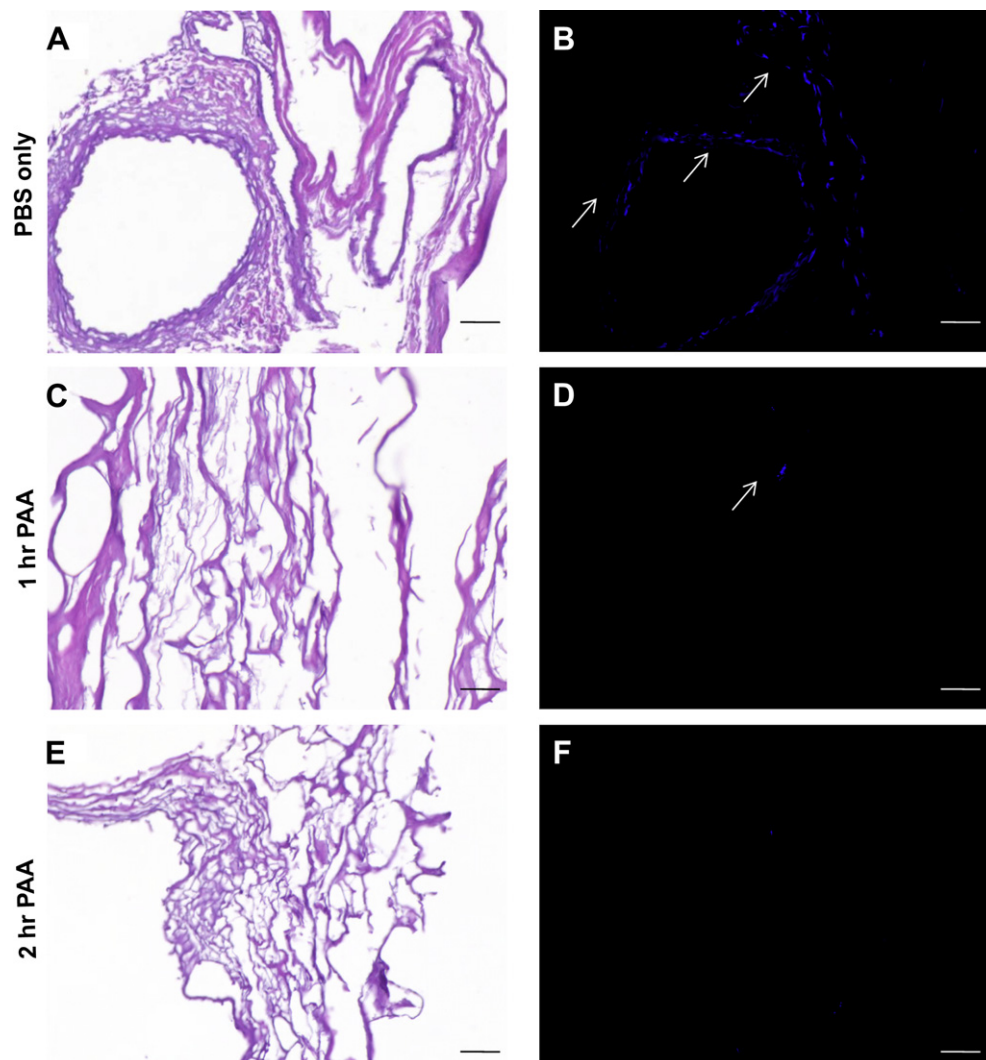


Fig. 1. Histologic images of SIS-ECM scaffolds. Decellularization of ECM devices was assessed histologically by hematoxylin and eosin (H&E) and 4',6-diamidino-2-phenylindole (DAPI) staining. Scaffolds prepared by the PBS protocol contained intact nuclei visible by H&E and DAPI staining (A,B). No intact nuclei were seen on scaffolds prepared following the 1 h PAA protocol and 2 h PAA protocol by H&E staining (C,E). While no DNA fragments were seen by DAPI staining in samples following the 2 h PAA protocol (F), small fragments were seen in scaffolds following the 1 h PAA protocol (D). Scale bar = 100 μ m.

three described methods of decellularization. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM Invitrogen) supplemented with 20% fetal bovine serum (FBS, Hyclone), 2 mM L-glutamine (Invitrogen), 100 units/ml penicillin, 100 µg/ml streptomycin (Invitrogen) at 37 °C in a humidified atmosphere with 5% CO₂.

At confluency, the cells were passaged and seeded onto 1.5 cm × 1.5 cm sheets of lyophilized SIS-ECM at a density of 1×10^6 cells/scaffold in 12-well plates ($N = 4$ per scaffold type). At 4 days post-seeding, the scaffolds were fixed with 4% paraformaldehyde and snap frozen in OCT embedding media for frozen sectioning.

2.5. In vivo macrophage response to SIS-ECM scaffolds

All procedures were performed in accordance with the National Institute of Health (NIH) guidelines for care and use of laboratory animals, and with approval of the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh. Twelve adult female Sprague–Dawley rats weighing approximately 300 g (Charles River Laboratory, Wilmington, MA) were randomly assigned to 3 separate groups ($N = 4$ /group) based on scaffold type: (1) acellular (2 hr PAA) (2) partially decellularized (1 hr PAA) and (3) cellular (PBS). A partial thickness abdominal wall defect model [34,37] was used to evaluate the host response to each scaffold type.

Each animal was anesthetized by inhalation with 2% isoflurane in oxygen, and the ventral abdominal wall was prepared for sterile surgery. A ventral midline abdominal skin incision was created, and the skin and subcutaneous tissue were separated from the underlying muscle tissues on one side of the midline for approx. 4 cm to the anterior axillary line. A 1 cm × 1 cm section of the external and internal oblique muscles on the ventral lateral abdominal wall, adjacent to the linea alba, was excised leaving the underlying transversus fascia and peritoneum intact. The defect was repaired with a size-matched piece of the chosen test scaffold. A single 4–0 Prolene suture was placed at each of the four corners of the test article to secure the device to the surrounding musculature, allow for mechanical loading of the test article during the normal abdominal wall activity of daily living, and to demarcate the implant for identification at the time of necropsy. A subcuticular placement of 4–0 Vicryl suture was used to close the skin incision. Each animal was recovered from anesthesia and was returned to the housing unit. Each animal was housed individually in shoebox cages. The rats were fed a diet of Purina Isopro rodent chow ad libitum (LabDiet ProLab Isopro RMH 3000, PMI Nutrition International, St. Louis, MO). The housing environment was maintained at 68° to 76 °C for 24 h a day and with a light:dark cycle of 12:12 h. Each rat received 0.02 mg/kg Buprenex (buprenorphine hydrochloride) by subcutaneous injection the day of surgery and for two additional days. Baytril (20 mg) was given orally the day of surgery and for two additional days. The dietary habits, general health status, and the surgical site were monitored daily and recorded.

Animals were sacrificed at 14 and 28 days post-surgery. Each rat was euthanized with 5% isoflurane in oxygen followed by an intracardiac injection of 5 mL of potassium chloride to induce cardiac arrest. The implant site, including the surrounding native abdominal wall tissue was excised, mounted on a fixed support structure to maintain the size and shape of the *in situ* tissue, and placed in 10% neutral buffered formalin (NBF). The sections were then processed for routine paraffin sectioning.

2.6. Immunolabeling

Immunolabeling of both *in vitro* and *in vivo* tissue samples was performed on 5 µm sections. Frozen sections were fixed in ice cold 50:50 acetone: methanol (v/v) for 5 min and rinsed 3 times in PBS. Paraffin embedded sections were deparaffinized with xylene and rehydrated through a graded ethanol series. Heat-mediated antigen retrieval was performed with 0.1 mM EDTA buffer at 95–100 °C for 25 min.

The tissue sections were subjected to Tris-Buffered Saline Tween-20 (TBST) for 15 min, followed by incubation in blocking buffer (1% bovine serum albumin/0.05% Tween-20/0.05% Triton X-100) for 1 h. The primary antibodies, diluted in blocking buffer, were added to the slides for 16 h at 4 °C in a humidified chamber. The slides were then washed three times in PBS prior to the addition of the secondary antibody for 1 h in a humidified chamber at room temperature. The primary antibodies used in this study were Alexa Fluor® 488 anti-mouse CD206 (1:50, BioLegend, San Diego, CA), goat polyclonal CD206 (Santa Cruz Biotech, Santa Cruz, CA), rabbit monoclonal CCR7 (1:100, Epitomics, Burlingame, CA) and mouse anti-rat CD68 (1:50, Serotec, Raleigh NC). The secondary antibodies used were Alexa Fluor® goat anti-rabbit 568 (Invitrogen), Alexa Fluor® donkey anti-goat 488 (Invitrogen) and Alexa Fluor® donkey anti-mouse 350 (Invitrogen). CD68 is a pan-macrophage marker. CCR7 is an M1 marker. CD206 is an M2 marker. For *in vitro* studies, 4', 6-diamidino-2-phenylindole (DAPI) was used as a nuclear counterstain. For *in vivo* studies, Draq5 (Cell Signaling Technologies, Danvers MA) was used as a nuclear counterstain by the manufacturers recommended protocol. All primary antibodies were confirmed to cross-react with either mouse or rat epitopes.

2.7. Quantification of M1/M2 macrophage staining

All tissue sections were imaged using a Zeiss Axio Observer Z1 microscope with appropriate brightfield and fluorescent filter sets. For analysis of *in vitro* macrophage

polarization, five ×320 magnification fields of view per scaffold were obtained at random intervals along the length of the scaffold. For analysis of *in vivo* macrophage polarization, ten random ×320 magnification fields of view were obtained which spanned the area between the proximal and distal sutures to the native tissue.

Quantification of M1/M2 polarization was achieved using a custom image analysis pipeline developed using the cell profiler image analysis package [44,45]. This custom pipeline identified and quantified the number of CD68+ CCR7+ (M1 phenotype) and CD68+ CD206+ (M2 phenotype) cells present within the tissue sections. Any cells that co-expressed these markers were not counted. These numbers were then expressed as a ratio of M1/M2.

2.8. Statistical Analysis

Analysis of variance was used to determine statistical significance of the difference in scaffold decellularization using M1/M2 ratio. The Tukey multiple comparison test was used to determine which pairs of decellularization methods were significantly different when a main effect was statistically significant. Where samples variances were found to be non-homogenous, the Kruskal–Wallis analysis was used as an alternative.

3. Results

3.1. DNA concentration and fragmentation in scaffolds

The amount of tissue decellularization following the three preparation methods was assessed using previously established

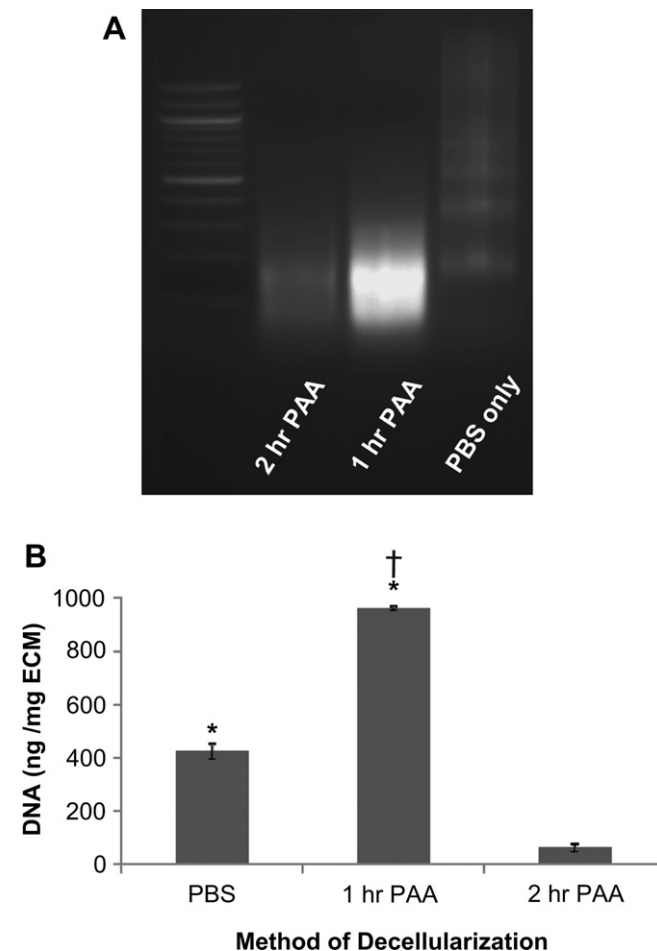


Fig. 2. Concentration of remnant DNA in ECM scaffolds. The amount of DNA remaining in the ECM scaffolds prepared by varying methods was quantified as a marker of remaining cellular material. Dagger symbol indicates ECM prepared using the 1 h PAA protocol contained the highest DNA concentration while, interestingly, ECM prepared using the PBS protocol showed lower levels of DNA. Asterisk indicates both of these levels were significantly higher ($p < 0.05$) than ECM prepared using the 2 h PAA protocol (B). DNA fragment length was assessed by gel electrophoresis using a reference 100 bp ladder (A).

guidelines for decellularization [26]. Intact nuclei were visible by H&E and DAPI staining on samples prepared with PBS washing only (Fig. 1A and B). No intact nuclei were seen by H&E staining on samples treated with PAA for either 1 hr (Fig. 1C) or 2 h (Fig. 1E) although potential fragments of DNA were seen attached to the ECM fibers in DAPI stained samples following 1 hr PAA protocol (Fig. 1D). No nuclear material was seen in samples following the 2 hr PAA protocol (Fig. 1F).

The concentration of remaining DNA within the ECM materials was used as a measure of remaining cellular material. ECM prepared using the 1 hr PAA protocol contained the greatest concentration of DNA with 959 ± 9.2 ng/mg ECM. Interestingly, ECM prepared using the PBS only protocol showed a lesser concentration of DNA ($p < 0.05$) with 423 ± 29.7 ng/mg ECM. Both of these concentrations were greater ($p < 0.05$) than those of the ECM prepared using the 2 hr PAA protocol which had an average DNA concentration of 62 ± 16.4 ng/mg ECM. The isolated DNA was assessed for fragment size using agarose gel electrophoresis. In

samples prepared using the 1 hr or 2 hr PAA protocols, all residual DNA was less than 500 bp in length. In contrast, scaffolds prepared using the PBS only protocol showed the residual DNA to range from 100 bp to greater than 1500 bp and likely included intact full length DNA (Fig. 2).

3.2. *In vitro* macrophage response

The response of macrophages to ECM scaffolds with differing amounts of residual DNA was assessed *in vitro* using the RAW 264.7 macrophage cell line. Immunolabeling for CCR7 (M1 marker) and CD206 (M2 marker) showed a distinct pattern of macrophage polarization depending on the method of ECM preparation. On scaffolds prepared using the PBS protocol $90.3\% \pm 16.9\%$ were CCR7+ (Fig. 3A). On ECM prepared using the 1 hr PAA protocol the phenotype of CCR7+ macrophages decreased to $55.0\% \pm 17.1\%$ (Fig. 3C). ECM prepared using the 2 hr PAA protocol showed only $25.2\% \pm 15\%$ of macrophages with a CCR7+ marker (Fig. 3E). The

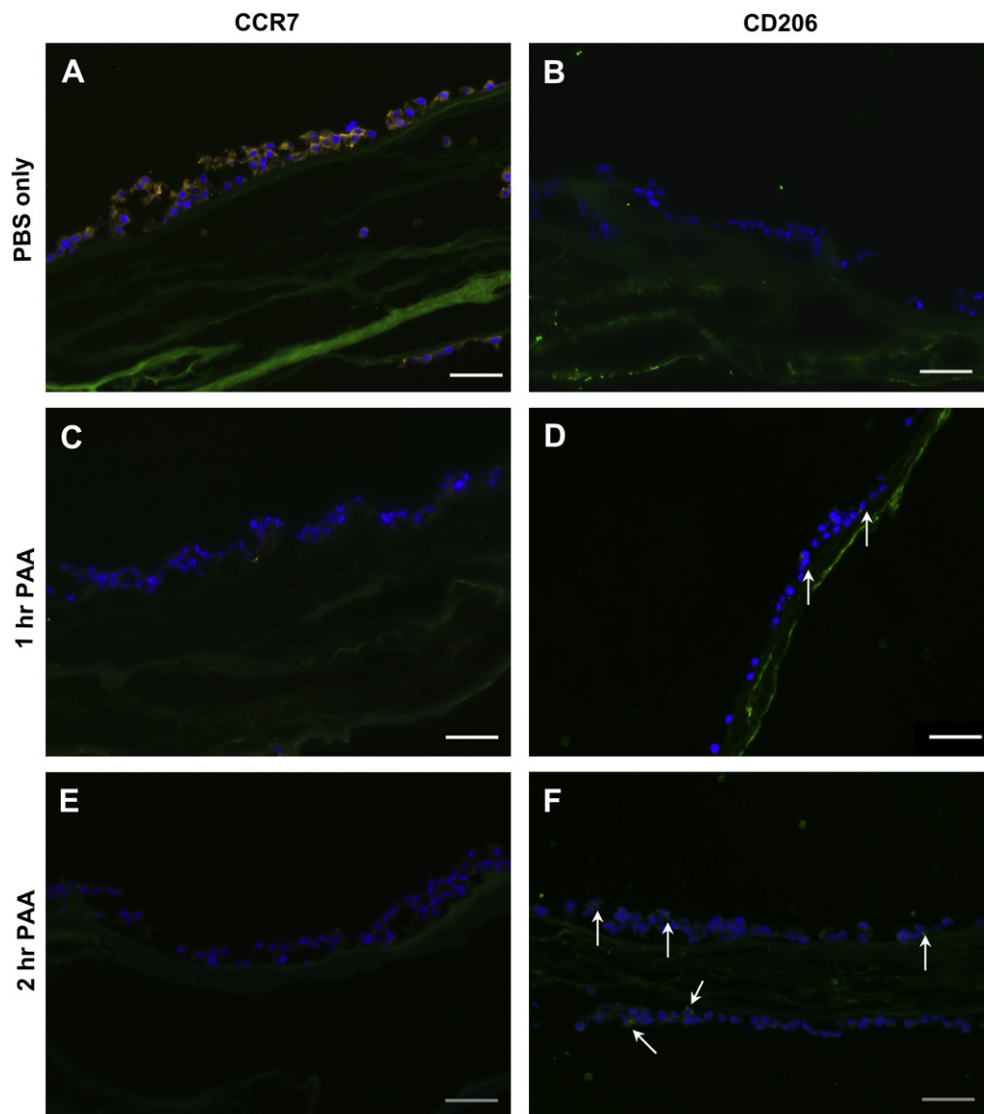


Fig. 3. Immunolabeling images of macrophage phenotype *in vitro*. The macrophage response to ECM scaffolds was assessed *in vitro* using the RAW 264.7 macrophage cell line. Immunolabeling for M1, CCR7+, macrophages (orange) and M2, CD206+, macrophages (green) showed that macrophages show distinct polarization profiles depending on the method of decellularization. On scaffolds prepared following the PBS and 1 hr PAA protocols, macrophages showed high levels of CCR7+ staining (A, C) and lower levels of CD206+ staining (B, D). However, macrophages responded with a predominant M2 phenotype on scaffolds prepared using the 2 hr PAA protocol with high levels of CD206+ staining (F) and little CCR7+ staining (E). Arrows indicate positive staining and scale bars = 50 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

percentage of CD206+ cells on each scaffold was $18.0\% \pm 9.2\%$ (Fig. 3B), $28.0\% \pm 15.9\%$ (Fig. 3D) and $46.0\% \pm 6.1\%$ (Fig. 3F) for scaffolds prepared using PBS, 1 hr PAA and 2 hr PAA, respectively. Since CD68+ macrophages were present on the scaffolds and some of these cells did not stain positive for surface markers indicative of polarization or may have co-expressed both markers, it is logical that the percentage of CCR7+ and CD206+ cells does not add to 100%. Calculation of the M1/M2 ratio for each ECM showed that scaffolds prepared using the PBS protocol promoted a strong M1 polarization while scaffolds prepared using the 1 hr PAA and 2 hr PAA protocols had a significantly lower ($p < 0.05$) M1/M2 ratio (Fig. 4).

3.3. Macrophage response in vivo

Four-layer multi-laminate scaffolds prepared with sheets of ECM from each of the decellularization protocols were implanted for 14 and 28 days in the rat partial thickness abdominal wall defect model. All of the animals survived the surgical procedure and post-operative period without complications. The host response to the implanted scaffolds was assessed by macroscopic examination and by both qualitative and quantitative histologic methods. Macrophage phenotype was characterized by immunolabeling methods. All scaffolds showed a robust macrophage presence that continued through 28 days although there were distinct differences in the density and distribution M1 vs. M2 phenotype dependent upon the decellularization method. All scaffold materials showed a significant decrease in the total number of cells between day 14 and day 28 (Fig. 7A) although the proportion of CD68+ cells remained approximately equal between each time point. CD68+ macrophages were present within the wound site, some of which did not stain positive for either M1 or M2 surface markers. These macrophages may have just arrived at the site and not yet express polarization towards an M1 or M2 phenotype. Similarly, other cells may have been co-expressing both markers and not counted by the cell profiler system. Therefore it is logical that the percentages of CCR7+ and CD206+ cells would not equal 100%.

3.4. Macrophage response to PBS-treated scaffolds

Devices prepared using the PBS protocol showed the highest density of cell infiltration at day 14 ($P < 0.05$) (Fig. 7A). Of these invading cells approx. $21.3\% \pm 0.05\%$ were CD68+ macrophages. Macroscopically the implant sites all showed swelling and seroma

formation that were not present in either the 1 hr PAA or 2 hr PAA-treated scaffolds. The mononuclear cells formed dense accumulations particularly within the middle of the implant site (Fig. 5A–B). A strong angiogenic response was present within the implant site. The device was clearly distinguishable and a dense layer of mononuclear cells surrounded the scaffold fibers. Immunolabeling studies showed that these scaffolds had the highest value for concentration of macrophages with the mean of 455 ± 82.7 CD68+ cells per field of view. However, this value was not statistically different from the 1 hr PAA or 2 hr PAA-treated scaffolds. The M1 macrophage phenotype predominated in a cell layer immediately surrounding the ECM scaffold (Fig. 5C), and M2 cells were mainly located near the suture line with the native tissue and in areas of remodeling peripheral to the ECM scaffold. Quantitative analysis of macrophage phenotype showed that the percentages of M1 and M2 over the entire implant site (scaffold plus adjacent tissue) were similar with $53.7 \pm 9.6\%$ and $57.4\% \pm 6.6\%$ respectively. As a result, the M1/M2 ratio slightly favored the M2 phenotype with a ratio of $0.93 \pm 0.17:1$.

By day 28 there was a decrease in total numbers of infiltrating cells ($P < 0.05$) with the remaining cells localized in dense accumulations surrounding the scaffold (Fig. 6 A and B) that was not observed in the 1 hr or 2 hr PAA-treated samples. Although total cell numbers decreased, the proportion of macrophages remained similar to day 14 ($19.3\% \pm 0.05\%$). Immunolabeling studies showed while there was no change in the M1/M2 ratio (1.01 ± 0.19) the proportion of M1 macrophages had increased and distribution of macrophages within the defect site had become more distinct than at day 14. The dense cell accumulations surrounding the scaffold were predominantly M1 macrophages (Fig. 6C) while M2 macrophages were located in areas of remodeling peripheral to the ECM scaffold.

3.5. Macrophage response to 1 hr PAA-treated scaffolds

Devices prepared using the 1 hr PAA protocol showed an intense cell response at 14 days which was markedly less than PBS-treated scaffolds, but similar to 2 hr-PAA treated scaffolds. Of these cells $22.6\% \pm 0.04$ were CD68+ macrophages. Macroscopically, there was a slight swelling at the implant site but no sign of seroma formation. Small and scattered remnants of the ECM device were visible and mononuclear cells were distributed more evenly throughout the individual layers of the multi-laminate device than was observed in the PBS only treated samples. A robust angiogenic response was present and there was abundant neomatrix deposition (Fig. 5D–E). Immunolabeling studies showed large numbers of CD68+ macrophages throughout the scaffold with an average of 395.4 ± 83.9 CD68+ cells per field. The macrophages surrounding the scaffold fibers predominately expressed the M1 marker CCR7. However, some CD206+ M2 macrophages were also observed immediately adjacent to the scaffold and the distribution of M2 cells was more uniform than was observed in the PBS-treated scaffold (Fig. 5F). Quantitative analysis of polarized macrophages over the entire implant site showed that M1 macrophage percentage was similar to that found in the PBS only treated devices with $56.3\% \pm 16.0\%$ CCR7+ and $65.0\% \pm 7.6\%$ CD206+ with an M1/M2 ratio of $1.02 \pm 0.23:1$.

By day 28 there was a decrease in total numbers of infiltrating cells ($P < 0.05$) with the remaining cells uniformly throughout the defect site although small clusters of cells were observed around the scaffold edges (Fig. 6 D and E). As with the PBS-treated scaffolds, the proportion of macrophages infiltrating the defect site remained similar to day 14 levels ($20.2\% \pm 0.04\%$). Immunolabeling studies showed no change in the M1/M2 ratio (0.89 ± 0.27) although the proportion of M2 macrophages had increased slightly.

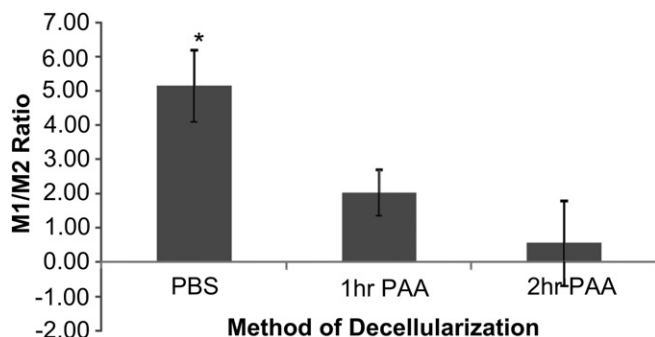


Fig. 4. Macrophage response *in vitro*. Calculation of the M1/M2 ratio of each ECM showed that scaffolds prepared using the PBS protocol promoted a strong M1 polarization while scaffolds prepared using the 2 h PAA promoted a predominant M2 phenotype. An M1/M2 value of 1 indicates equal amounts of cells expressing CCR7 and CD206. An M1/M2 ratio above 1 indicates an M1 majority and values below 1 indicates an M2 majority. Asterisk indicates significantly higher M1/M2 ratio ($p < 0.05$) in scaffolds decellularized by PBS method relative to the other two methods for decellularization.

Macrophages were distributed more uniformly throughout the defect site than in the PBS-treated scaffolds although M1 macrophages still predominated immediately around the remaining scaffold material. (Fig. 6F).

3.6. Macrophage response to 2 hr PAA-treated scaffolds

Devices prepared using the 2 hr PAA protocol showed a cell response at 14 days, similar to 1 hr PAA-treated scaffolds and significantly lower than PBS-treated scaffolds (Fig. 7A). Of these cells $22.6\% \pm 0.39\%$ were CD68+ macrophages. Macroscopically there was no sign of swelling or seroma. Histologic examination showed mononuclear cells present throughout the scaffold and between layers of the multi-laminate device similar to the 1 hr PAA treatment (Fig. 5G–H). Remnants of the device were still visible along with deposition of a neomatrix and a strong angiogenic response. Immunolabeling studies showed that these scaffolds had similar numbers of macrophages to the 1 hr PAA scaffolds with an average of 407 ± 100.7 CD68+ cells per field. The spatial distribution of polarized macrophages showed that M1 macrophages were still the predominant phenotype immediately adjacent to the scaffold fibers. However, M2 macrophages were more evenly distributed throughout the implant site rather than being limited to the periphery of the implant site as was seen with the PBS-treated scaffolds (Fig. 5I). Quantitative analysis of M1 and M2 phenotype showed no difference to either the PBS or 1 hr PAA-treated scaffolds

with an average $53.7\% \pm 15.8\%$ of macrophages expressing the M1 marker and $61.2\% \pm 7.6\%$ of macrophages expressing the M2 marker. Overall the M1/M2 ratio slightly favored the M2 phenotype with a ratio of $0.87 \pm 0.22:1$ although this value was not significantly different to the values for either the 1 hr PAA or PBS only treated scaffolds (Fig. 6).

By day 28 there was a decrease in total numbers of infiltrating cells ($P < 0.05$) with the remaining cells mainly distributed throughout the defect site (Fig. 6 G and H). The proportion of macrophages infiltrating the defect site remained similar to day 14 levels ($22.5\% \pm 0.05\%$). There was no change in the M1/M2 ratio (0.94 ± 0.21) although compared to the 1 hr PAA- and PBS-treated scaffolds the cells were distributed uniformly throughout the defect site and the dense accumulations of M1 macrophages were not observed (Fig. 6).

4. Discussion

The present study attempts to determine the association between decellularization efficacy and host response by qualitative and quantitative methods. Quantitative criteria of decellularization have not been described until recently [26]. The presence of xenogeneic DNA within biologic scaffold materials has been suggested as a possible cause of an “inflammatory response” [20] in patients. Indeed, many commercial biologic scaffolds contain varying amounts of remnant DNA [28,46]. This remnant DNA is

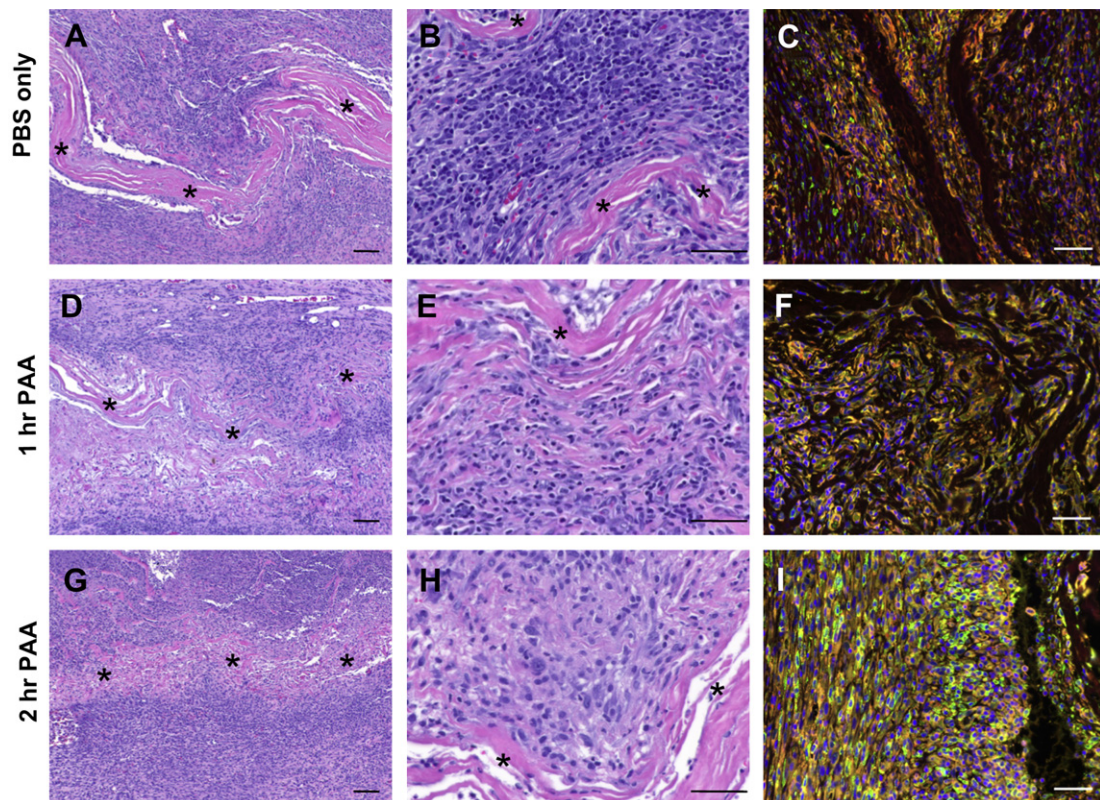


Fig. 5. Histologic and immunolabeling images of the defect site at 14 days post-surgery. The *in vivo* host response to scaffolds prepared using varying methods of decellularization was assessed histologically with hematoxylin and eosin (H&E) staining and by the immunolabeling of macrophages. Devices prepared using the PBS protocol (A–C) showed the highest levels of mononuclear cell infiltration (A & B) with M1 cells (orange) predominating in a layer surrounding the ECM scaffold (C), while M2 cells were mainly located in areas surrounding the ECM scaffold close to the suture line with the native tissue. Devices prepared using the 1 hr PAA protocol (D–F) showed a similar distribution of macrophages (D & E), however, some M2 cells were observed surrounding the scaffold and the M2 cell distribution was more even than the PBS-treated scaffold (F). The polarized macrophages in the 2 hr PAA-treated scaffolds (G–I) showed that while M1 macrophages still predominated around the scaffold fibers, M2 macrophages were more evenly and widely distributed throughout the scaffold rather than being limited to the margins of the wound as seen with PBS scaffolds (I). Immunolabeling showed the presence of macrophages (red) in all scaffolds and used DRAQ5 (blue) as a nuclear stain. Scale bars = 50 μ m, * = scaffold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

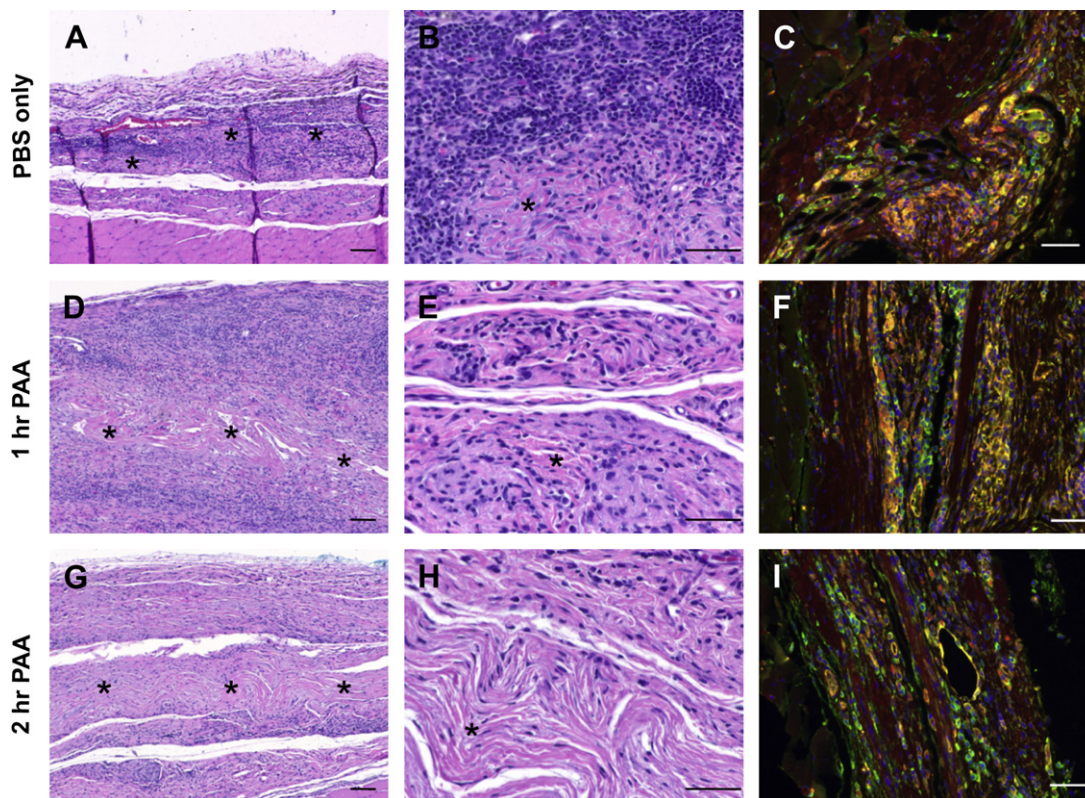


Fig. 6. Histologic and immunolabeling images of the tissue surrounding implant site at 28 days post-surgery. Devices prepared using the PBS protocol (A–C) maintained a high cellular response at 28 days post-surgery (A) with dense accumulations of mononuclear cells surrounding scaffold (B). Devices prepared using the 1 hr PAA protocol (D–F) and 2 hr PAA protocol (G–I) contained mononuclear cells with more even distribution. Characterization of the distribution of polarized macrophages in the scaffolds showed that M2 cells (green) was more uniform throughout the 2 hr PAA scaffolds compared to the 1 hr PAA scaffold and PBS scaffold. The PBS scaffold and 1 h PAA scaffold contained clusters of M1 (orange) macrophages that were not observed in the 2 hr PAA treatment. Immunohistochemical staining showed the presence of macrophages (red) in all scaffolds and used DRAQ5 (blue) as a nuclear stain. Scale bars = 50 μ m, * = scaffold remnants. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

typically present as small fragments less than 300 bp in length [28], and this remnant DNA is therefore unlikely to play any substantive role in an adverse tissue remodeling response [47]. However, the question remains as to whether there is a threshold level of cellular “debris” that will elicit an adverse remodeling outcome [20].

The current study utilized SIS-ECM as the test article, since it is one of the most comprehensively studied matrices with regard to biological activity, composition, mechanical properties and macro- and ultra-structure [46,48–52]. In addition, a wealth of studies have characterized the host constructive remodeling response to this material [2,14,18,25,42,53–55]. The relative ease of preparation, compared to other biologic scaffold materials, allowed careful control of the decellularization procedures to ensure consistency in the extent of decellularization between batches. This consistency was confirmed by the fact that DNA concentration varied by less than 30 ng/mg ECM in each decellularization group.

The negative effects of the presence of intact allogeneic or xenogeneic cells within a biologic scaffold are widely recognized; therefore, it is logical that the effective removal of antigenic epitopes and intracellular components of source tissues and organs is necessary to minimize an adverse immune response by recipients of an ECM scaffold device. It has been shown that the presence of cells within a scaffold is associated with increased amounts of proinflammatory cytokines, increased macrophage M1 polarization, and a poor remodeling outcome in a primate model [56]. Previous studies have shown that the implantation of a xenogeneic ECM scaffold containing a cellular component results in the classic cascade of inflammatory processes with mononuclear cell

infiltration, including macrophages predominantly of an M1 phenotype at 3 days post implantation, with eventual scar tissue formation [36]. Furthermore, when the same scaffold materials are prepared by methods that remove the cellular component, the mononuclear cell response is marked by macrophages primarily of an M2 phenotype [36].

The current study used DNA content as a quantitative marker of cellular remnants. The decellularization protocols developed resulted in three distinct levels of decellularization; one which retained intact cells and full length DNA, one with large amounts of small fragment DNA, and one that contained small amounts of small fragment DNA. While all three protocols were effective at removing or lysing intact cells and cellular material, even within the PBS alone protocol, the aggressiveness of the decellularization process had a marked *in vitro* effect on macrophage polarization and was associated with a more favorable macroscopic host tissue response *in vivo*, including less swelling and absence of seroma formation. While ECM treated with the 1 hr PAA protocol contained a greater amount of DNA compared to the PBS-treated ECM, more M2 macrophage polarization was seen suggesting that DNA content alone is not the sole determinant of the host response. This study also demonstrated that PAA treatment, rather than mechanical agitation, is the key factor in the dissociation of DNA and likely other cellular components from the remaining ECM. The dissociated cell remnants can then be effectively removed by the mechanical agitation process. It is important to note that DNA was used in this study as an indicator of the presence of cell remnants and it is almost certain that other cell components capable of

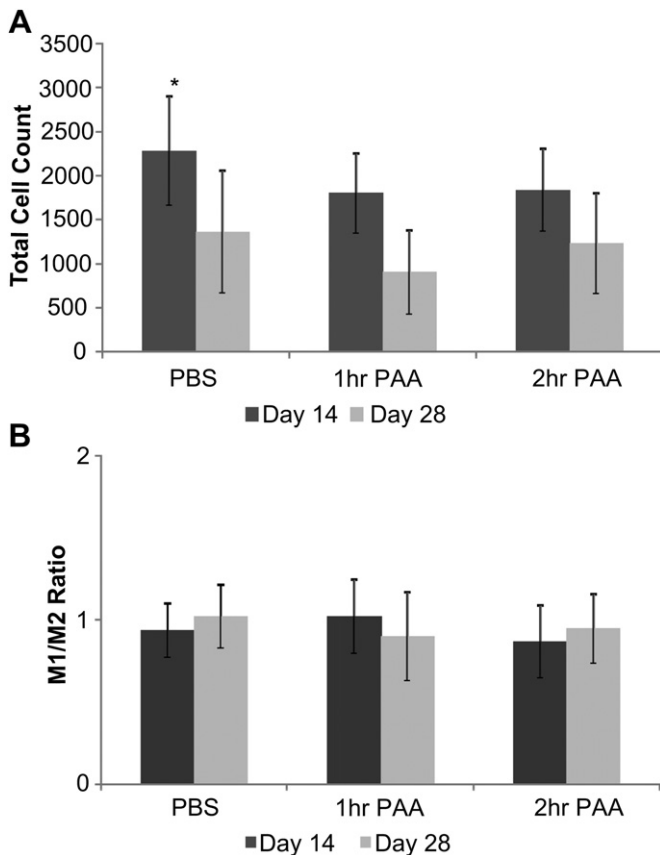


Fig. 7. Cellular response to ECM scaffolds *in vivo* at 14 and 28 days. All treatment groups showed a significant decrease in numbers of infiltrating cells between days 14 and 28 (A). PBS-treated scaffolds showed highest level of cellular infiltration a day 14 compared to 1 hr PAA and 2 hr PAA scaffolds (* = $P < 0.05$). M1/M2 macrophage ratio in response to devices prepared using the PBS and 2 hr PAA protocols showed the macrophage response favored M2 while the macrophage phenotype on devices prepared using the 1 hr PAA protocol favored M1 slightly at 14 days (B). However, no treatment resulted in a significantly different macrophage profile and no change on M1/M2 ratio was seen at day 28. An M1/M2 value of 1 indicates equal amounts of cells expressing CCR7 and CD206. M1/M2 ratios above 1 indicate an M1 majority and below 1 is an M2 majority.

causing an adverse response are retained in the scaffold through the process of decellularization. For example, mitochondria are known to release damage associated molecular patterns (DAMPs) that promote a proinflammatory response [41]. It is unknown if DNA quantification is the best indicator, or even a good indicator, of “decellularization” but alternative sensitive and quantitative methods of assessing the presence of cell remnants were not available or used in this study. The results of the present study clearly show that less complete removal of the cell remnants and increased DNA fragment size are associated with a more proinflammatory macrophage phenotype *in vitro* and that more thorough removal of cell remnants and effective fragmentation of cellular material are associated with a more diffuse and widespread distribution of the constructive M2 phenotype.

While the present study used three decellularization protocols that were based on variations of the standard method for SIS-ECM preparation [42,49], other studies have investigated the effects of different decellularization methods. Kasimir et al showed that certain enzymes or detergents could cause structural alterations to the matrix and produce varying degrees of decellularization [57]. Similarly, Meyer et al demonstrated that the decellularization method influenced the immune reactivity of aortic valve tissue with tissue prepared with hypertonic or hypotonic buffer washes,

similar to the PBS protocol of the present study, exhibiting a strong anti-MHC response while detergent treatments significantly reduced antigenicity [58]. Other studies have also shown varying success rates with different decellularization methods [59,60], although none of these studies used any strict criteria for assessing decellularization other than the absence of intact cells.

Analysis of the macrophage response to the biological scaffold materials in the current study *in vitro* examined the response of macrophages at a relatively early time point. The devices containing significantly more cellular material, prepared by the PBS only and 1 hr PAA protocol, showed a predominantly M1 macrophage response while the device containing less amounts of DNA, prepared using the 2 hr PAA protocol, resulted in a macrophage response predominantly of an M2 phenotype. This data supports the findings of previous studies using an *in vivo* model where a cellular scaffold promoted a clear M1 phenotype macrophage response at 3 days whereas the equivalent acellular scaffold promoted a strong M2 phenotype [36]. Rieder et al have shown that decellularization can reduce the chemotactic potential of heart valve tissue for macrophages but does not inhibit the activation of macrophages although they did not study macrophage polarization [60]. In addition, Ariganello et al. have shown that *in vitro* exposure of a macrophage cell line to decellularized tissue elicited lower esterase and phosphatase activity consistent with a subdued inflammatory response comparable to the M2 phenotype observed in the present study [35].

Interestingly, the significant differences seen with the *in vitro* assays were not quantitatively apparent when the macrophage response *in vivo* was evaluated at 14 and 28 days after implantation. All the scaffold materials elicited a mixed macrophage response with no significant differences observed in total number of macrophages or M1/M2 ratio. The notable and perhaps more important differences were found in the distribution of macrophages within the various scaffolds. Scaffolds treated with only PBS contained dense accumulations of M1 cells within the scaffolds and especially at the scaffold interface with native tissue whereas the M2 macrophages were mainly located peripheral to the ECM scaffold. As the method for decellularization became more aggressive, M1 macrophages still were the dominant phenotype immediately adjacent to the scaffold fibers but the distribution of M2 cells became more uniform and widely distributed throughout the implant site.

The results at day 28 showed a significant decrease in the total number of infiltrating cells but no difference in proportion of macrophage cells or M1/M2 ratio. This decrease in total cell numbers is consistent with the normal wound healing process that is characterized by a period of cell migration and proliferation during the early stages (7–10 days) [39] followed by reduction of cell density concomitant with tissue remodeling [39,61]. Previous studies have shown that the macrophage response is regulated in part by cytokines release as a consequence of injury and the healing response [30,38,62,63]. In the present study, a prolonged M1 response was observed surrounding the PBS-treated scaffold at day 28 suggesting that cellular remnants within this scaffold were still stimulating a proinflammatory response at day 28. Conversely, the macrophages within the 2 hr PAA-treated scaffolds were much more evenly distributed, suggestive of a diminished proinflammatory response to the scaffold material. It is unknown whether certain regions, such as the interface between the ECM implant and the native tissue, are a more important determinant of downstream tissue remodeling outcomes than regions more peripheral to the implant. In addition, macrophage phenotype may not be a strong indicator of remodeling outcomes at all time points, particularly later time points, and it may be necessary to examine macrophage phenotypes only at times shortly after surgery to predict the remodeling outcome.

There were several limitations in the present study. Only 2 time points were examined, 14 and 28 days after implantation. The 14 day time point was examined because it has been shown to be a useful time point for identifying changes in M1/M2 ratio in previous studies with this model [11,36,64], while day 28 represented a point at which the inflammatory response should be ending and transitioning to a constructive remodeling response [12,36]. Another limitation was that macrophage phenotype was determined solely upon cell surface markers. The present study used CCR7 and CD206 as markers of M1 and M2 macrophages respectively. Neither is specific for macrophages alone and co-staining with CD68 was used to identify polarized macrophages. However, the polarized macrophages can be subdivided into a number of subgroups [30] and these subgroups may not have been accurately counted using just two markers. A more complete picture of macrophage phenotype may have been provided by concomitant cytokine analysis. Previous studies have shown that SIS-ECM can affect systemic cytokine levels stimulating a Th2 response in experimental animals consistent with a tissue remodeling response, but does not cause generalized immune dysfunction [65,66]. Finally, any systemic effect of the ECM scaffolds was not accessed and only the local tissue response was evaluated. Circulating DNA has been associated with clinical signs of sepsis [67], may be the cause of a sepsis-like systemic response [68], and is present in patients with chronic inflammatory diseases [69]. Although there are limitations, this study represents a systematic effort to determine the relationship between decellularization efficacy and host response to the implantation of a biologic scaffold material.

5. Conclusion

The results of this study show that decellularization efficacy of biologic scaffold materials is at least one determinant of the macrophage phenotype response. Although a cause-effect relationship between macrophage phenotype and remodeling outcome has not been definitely shown, a clear association exists. Effective decellularization remains an important component in the production of ECM-based scaffolds for therapeutic applications.

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