

Solubilized extracellular matrix bioscaffolds derived from diverse source tissues differentially influence macrophage phenotype

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Received 15 October 2015; revised 1 July 2016; accepted 6 July 2016

Published online 21 September 2016 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.35894

Abstract: The host response to biomaterials is a critical determinant of their success or failure in tissue-repair applications. Macrophages are among the first responders in the host response to biomaterials and have been shown to be predictors of downstream tissue remodeling events. Biomaterials composed of mammalian extracellular matrix (ECM) in particular have been shown to promote distinctive and constructive remodeling outcomes when compared to their synthetic counterparts, a property that has been largely attributed to their ability to modulate the host macrophage response. ECM bioscaffolds are prepared by decellularizing source tissues such as dermis and small intestinal submucosa. The differential ability of such scaffolds to influence macrophage behavior has not been determined. The present study determines the effects of ECM bioscaffolds derived from eight different source tissues upon macrophage surface marker expression, protein content, phagocytic capability, metabolism, and antimicrobial activity. The results show that macrophages exposed to small intestinal submucosa (SIS), urinary bladder matrix (UBM), brain ECM (bECM), esophageal

ECM (eECM), and colonic ECM (coECM) express a predominant M2-like macrophage phenotype, which is pro-remodeling and anti-inflammatory (iNOS⁻/Fizz1⁺/CD206⁺). In contrast, macrophage exposure to dermal ECM resulted in a predominant M1-like, pro-inflammatory phenotype (iNOS⁺/Fizz1⁻/CD206⁻), whereas liver ECM (LECM) and skeletal muscle ECM (mECM) did not significantly change the expression of these markers. All solubilized ECM bioscaffold treatments resulted in an increased macrophage antimicrobial activity, but no differences were evident in macrophage phagocytic capabilities, and macrophage metabolism was decreased following exposure to UBM, bECM, mECM, coECM, and dECM. The present work could have important implications when considering the macrophage response following ECM implantation for site-appropriate tissue remodeling. © 2016 Wiley Periodicals, Inc. *J Biomed Mater Res Part A*: 105A: 138–147, 2017.

Key Words: biologic scaffold, bioscaffold, macrophages, extracellular matrix, host response

How to cite this article: Dziki JL, Wang DS, Pineda C, Sicari BM, Rausch T, Badylak SF. 2017. Solubilized extracellular matrix bioscaffolds derived from diverse source tissues differentially influence macrophage phenotype. *J Biomed Mater Res Part A* 2017;105A:138–147.

INTRODUCTION

The use of biomaterials for the repair or reconstruction of damaged or diseased tissues is commonplace across a wide range of clinical applications. The success (i.e., safety and efficacy) or failure of these biomaterials is dependent, in large part, upon the host tissue response following implantation. The host innate immune response, especially the macrophage response, is a critical determinant of downstream tissue remodeling outcomes. Macrophages represent a cell population with heterogeneous phenotypes that are involved in a variety of biologic processes including tissue homeostasis, inflammation, disease progression, and functional tissue reconstruction. Macrophages are among the first responders to pathogens, tissue injury, and also, implanted biomaterials.^{1–4} Macrophage phenotypes have been classified along a

spectrum ranging from M1 or pro-inflammatory cells to M2 or pro-healing and regulatory cells. These various phenotypes can be distinguished by cell surface markers, associated cytokines and effector molecules, and functional activity including nitric oxide (NO) production or ornithine synthesis, respectively,^{5,6} among others.

Biomaterials have been manufactured from both synthetic and biologic substrates and each has their associated advantages and disadvantages.⁷ Of relevance to the present study, biologic scaffolds composed of mammalian extracellular matrix (ECM) are associated with a constructive remodeling outcome following injury, as opposed to fibrosis or the classic foreign body reaction, and have been used in many clinical applications including dermal,^{8–10} cardiac,¹¹ musculoskeletal,^{12–15} and gastrointestinal repair.¹⁶ The host macrophage

Additional Supporting Information may be found in the online version of this article.

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TABLE I. Overview of Decellularization Protocols. Each of the ECM Bioscaffolds Tested Have Been Previously Decellularized and Characterized in the Indicated Reference With Unique Decellularization Protocols

ECM Bioscaffold	Decellularization Method	Reference
Small intestinal submucosa (SIS)	Mechanical removal of muscular layers followed by 0.1% peracetic acid and water washes	69
Urinary bladder matrix (UBM)	Mechanical removal of surrounding muscular layers followed by 0.1% peracetic acid and water washes	28
Skeletal muscle ECM (mECM)	Mechanical removal of fat and connective tissue, washes with 2:1 (v/v) chloroform:methanol, graded ethanol series, 0.02% trypsin/0.05% EDTA, 2% deoxycholate, 1% Triton X-100, 0.1% peracetic acid, and water	33
Brain ECM (bECM)	Mechanical removal of dura matter, water wash, washes with 0.02% trypsin/0.05% EDTA, 3.0% Triton X-100, 1.0 M sucrose, 4.0% deoxycholate, and 0.1% peracetic acid	30
Esophageal ECM (eECM)	Mechanical removal of muscularis layer, washes with 1% trypsin/0.05% EDTA, 1.0 M sucrose, 3% Triton X-100, 10% deoxycholate, 0.1% peracetic acid, 4% ethanol, 100 U/mL DNase, water, and PBS	31
Dermal ECM (dECM)	Mechanical removal of fat, connective tissue, and epidermis, washes with 0.25% trypsin, 70% ethanol, 3% H ₂ O ₂ , 1% Triton X-100, 0.26% EDTA/0.69% Tris, water, 0.1% peracetic acid/0.4% ethanol, and PBS	70
Liver ECM (LECM)	Mechanical slicing/massage to aid cell lysis followed by washes with 0.02% trypsin/0.05% EDTA, 3% Triton X-100, 4% deoxycholate, and water	55
Colonic ECM (coECM)	Mechanical isolation of submucosa, washes with 2:1 (v/v) chloroform:methanol, graded ethanol series, 0.02% Trypsin/0.05% EDTA, 4% deoxycholate, 0.1% peracetic acid/4% ethanol, and water	71

phenotypic response was shown to be a determining factor in ECM bioscaffold-mediated remodeling outcomes.¹⁷ In turn, scaffold preparation and processing methods were shown to have a profound influence upon macrophage phenotype in a study utilizing small intestinal submucosa (SIS) ECM as a body wall repair device.¹⁸ Subsequent studies have confirmed and expanded the importance of macrophage phenotype in biomaterial-mediated tissue remodeling.^{19–25} Specifically, it has been shown that the process of ECM-mediated tissue remodeling relies upon the infiltration and activation of host macrophages toward an immunomodulatory, M2-like phenotype^{3,4} and that a higher M2/M1 ratio at early time points is indicative of a favorable, constructive remodeling outcome at later time points.³ Although it has been shown that pepsin-solubilized SIS-ECM bioscaffolds activate macrophages toward an M2-like phenotype, with increased Fizz1 and CD206 expression,²⁶ the mechanisms by which ECM bioscaffolds directly affect macrophage phenotype remain poorly understood.

ECM bioscaffolds have been prepared from many source tissues including small intestine,²⁷ urinary bladder,²⁸ brain,^{29,30} esophagus,³¹ liver,³² skeletal muscle,³³ and dermis,³⁴ among others, but the differential effects of these bioscaffolds upon macrophage phenotype have not been characterized. The objective of the present study is to characterize the phenotype of macrophages exposed to a variety of ECM scaffold materials,

each of which is derived from a different source tissue. Phenotypic analysis includes surface marker profile, protein expression, viability, metabolic activity, phagocytic capacity, and antimicrobial activity. The findings of this study may influence the choice of ECM bioscaffolds for clinical use.

METHODS

Overview of experimental design

ECM bioscaffolds were prepared from porcine tissues utilizing established protocols in accordance with previously established decellularization guidelines.³⁵ Murine bone-marrow derived macrophages were treated with pepsin-solubilized ECM bioscaffolds, and then harvested to analyze surface marker expression via immunolabeling, protein expression via western blotting, cell integrity by trypan blue exclusion, MTT metabolism, phagocytic capability, and antimicrobial activity.

Preparation of solubilized ECM bioscaffolds

Biologic scaffolds composed of porcine small-intestinal submucosa (SIS),²⁷ urinary bladder matrix (UBM),²⁸ skeletal muscle ECM (mECM),³³ brain ECM (bECM),³⁰ esophageal ECM (eECM),³¹ dermal ECM (dECM),³⁴ liver ECM (LECM),³² and colon ECM (coECM) were prepared following previously established decellularization protocols (Table I). All scaffold materials met stringent requirements for sufficient decellularization;

TABLE II. Summary of Findings. Macrophage Phenotype Was Characterized by Surface Marker Expression, Protein Expression, Metabolism, Phagocytosis, And Antimicrobial Activity. In General, Small Intestinal Submucosa (SIS), Brain ECM (bECM), Esophageal ECM (eECM), and Colonic ECM (coECM), Promoted a Shift Toward an Anti-inflammatory M2-like Macrophage Phenotype Whereas Dermal ECM (dECM) Promoted a Slight Shift Toward a Pro-inflammatory, M1-like Macrophage Phenotype. Urinary Bladder Matrix (UBM), Skeletal Muscle ECM (mECM), and Liver ECM (LECM) Did Not Promote a Significant Shift Toward Either Macrophage Phenotype Based Upon the Evaluated Parameters in This Study (key: + = significantly increased, – significantly decreased compared to untreated control).

Macrophage Treatment	Immunolabeling (iNOS and Fizz1)	Western Blotting (iNOS and CD206)	Metabolic Activity (MTT)	Phagocytosis	Antimicrobial Activity
IFN γ + LPS	iNOS +	iNOS+	No change	No change	+
IL-4	Fizz1+	CD206+	No change	No change	+
Small intestinal sub-mucosa (SIS)	Fizz1+	iNOS–/CD206+	No change	No change	+
Urinary bladder matrix (UBM)	No change	CD206+	–	No change	+
Skeletal muscle ECM (mECM)	No change	No change	–	No change	+
Brain ECM (bECM)	Fizz1+	iNOS–	–	No change	+
Esophageal ECM (eECM)	Fizz1+	iNOS–/CD206+	No change	No change	+
Dermal ECM (dECM)	iNOS+	No change	–	No change	+
Liver ECM (LECM)	No change	No change	No change	No change	+
Colonic ECM (coECM)	Fizz1+	CD206+	–	No change	+

specifically, no visible intact nuclei by DAPI and hematoxylin and eosin (H&E) staining, remnant DNA concentration <50 ng/mg total scaffold dry weight, and DNA fragment length <200 base pairs.³⁵ Scaffolds were lyophilized and milled to form a particulate powder. The powder was then solubilized with pepsin as previously described for the preparation of an ECM hydrogel to yield a 10 mg/ml solution of solubilized ECM.³⁶ The solubilized ECM was then neutralized by addition of one-tenth digest volume of 0.1N NaOH and one-ninth digest volume of 10 \times PBS to bring pH to 7.4, phosphate buffer to 0.01M, and sodium chloride concentration to 0.15M.

SDS PAGE analysis

Protein composition of each of the solubilized ECM from the various tissues was compared qualitatively using SDS PAGE and a See Blue Pre-stained Molecular Weight Marker (Invitrogen). Five microgram of each ECM was added to a 4–20% polyacrylamide gel and run at 120 V for 2 h. The gels were stored in fixing buffer overnight and then stained with a Pierce Silver Stain for Mass Spectrometry Kit (Life Technologies) following the manufacturer's instructions.

Macrophage isolation and polarization

Mouse bone marrow was harvested as previously described.^{26,37} Briefly, female 6–8 week old C57bl/6 mice (Jackson Laboratories, Bar Harbor, ME) were euthanized via CO₂ inhalation and cervical dislocation. Aseptically, the skin from the proximal hind limb to the foot was removed, the tarsus and stifle disarticulated, and the tibia isolated. The coxofemoral joint was disarticulated for isolation of the femur. After removal of excess tissue, bones were kept on ice and rinsed in a sterile dish containing macrophage complete medium consisting of DMEM (Gibco, Grand Island, NY), 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad,

CA), 10% L929 supernatant,³⁷ 50 μ M beta-mercaptoethanol (Gibco), 100 U/mL penicillin, 100 μ g/mL streptomycin, 10 mM non-essential amino acids (Gibco) and 10 mM hepes buffer. The ends of the bones were transected and the marrow cavity was flushed with complete medium to collect bone marrow. Cells were washed, plated at 2 \times 10⁶ cells/mL, and allowed to differentiate into macrophages for 7 days at 37°C, 5% CO₂ with complete medium changes every 48 h as previously described.³⁸ After 7 days, resulting naïve macrophages were treated with basal medium consisting of 10% FBS, 100 μ g/mL streptomycin, 100 U/mL penicillin in DMEM and one of the following conditions as previously described: (1) 20 ng/mL IFN γ and 100 ng/mL of LPS to promote an M1-like phenotype, (2) 20 ng/mL IL-4 to promote an M2-like phenotype, (3) 200 μ g/mL of pepsin control buffer, or (4) 200 μ g/mL of ECM for 18 h at 37°C, 5% CO₂.³⁹

Immunolabeling

After 18 h, macrophages were washed and fixed with 2% paraformaldehyde. Following PBS washes, cells were incubated in blocking solution consisting of 0.1% Triton-X 100, 0.1% Tween 20, 4% normal goat serum, and 2% bovine serum albumin (BSA) for 1 h at room temperature to prevent non-specific antibody binding. The following primary antibodies were diluted in blocking solution: (1) monoclonal anti-F4/80 (Abcam, Cambridge, MA) at 1:200 dilution for a pan-macrophage marker, (2) polyclonal anti-iNOS (Abcam, Cambridge, MA) at 1:100 dilution for an M2 marker, and (3) polyclonal anti-Fizz1 (Peptrotech, Rocky Hill, NJ) for an M2 marker.^{40–42} Cells were incubated in primary antibodies for 16 h at 4°C. After PBS washes, cells were incubated in fluorophore-conjugated secondary antibodies (Alexa Fluor donkey anti-rat 488 or donkey anti-rabbit 488,

Invitrogen) for 1 h at room temperature. After PBS washes, nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) prior to imaging three 200 \times fields containing on average 900 cells each, using a live-cell microscope. Light exposure times were standardized to a negative isotype control and kept constant across images. Images were quantified utilizing CellProfiler Image Analysis software to obtain positive F4/80, iNOS, and Fizz1 percentages.

Western blotting

After treatment with cytokines or ECM, macrophages were lysed for western blot analysis. Cell lysates were diluted 1:1 in 2 \times Laemmli sample buffer with 5% beta-mercaptoethanol. Twenty microgram of protein was loaded per well in 4–20% Bio-Rad Mini-PROTEAN TGX Stain-Free polyacrylamide gels. Gels were run at 100 V for 15 min and then 150 V in 1X running buffer (30.3 g Tris, 144 g glycine, 10 mL 10% SDS solution in water). Separated proteins were transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes for 3 h at 150 mA in transfer buffer (10% 10 \times running buffer, 20% methanol, 70% water). The membranes were then incubated in blocking buffer (5% BSA and 0.1% Tween-20 in TBS) for 1 h to prevent non-specific antibody binding. Membranes were incubated in primary antibodies for 16 h at 4 $^{\circ}$ C at a 1:500 dilution for M1-like iNOS (abcam, Cambridge, MA) and M2-like CD206 (abcam) in 0.5% BSA and 0.1% Tween-20 in TBS. Membranes were washed 0.1% Tween-20 in TBS and incubated with horseradish peroxidase secondary antibodies applied at 1:5000 in 0.5% BSA and 0.1% Tween-20 in TBS. Five milliliter Bio-Rad Western ECL solution (Bio-Rad, Hercules, MA) was applied to each membrane for 5 min before imaging. Densitometry was calculated with ImageJ software.

Cell viability

Macrophage viability following treatment with ECM was evaluated using the trypan blue exclusion assay.⁴³ Exposed macrophages were washed with PBS and harvested with Accutase $^{\circ}$ (Stem Cell Technologies, Vancouver, CA) solution for 10 min, followed by inactivation with medium containing 10% FBS. Trypan blue solution (0.4% w/v) was mixed in a proportion 1:1 with the cell suspension. The number of viable cells (trypan blue excluded) and non-viable cells (trypan blue included) were determined using a hemocytometer. The percentage of cells showing trypan blue exclusion was determined dividing the amount of trypan blue excluding cells by the total number of cells.

Macrophage metabolism

Metabolism of exposed macrophages to the tissue-specific ECM was measured using the MTT assay (Vibrant $^{\circ}$ MTT Cell Proliferation Assay Kit, V-13154, Molecular Probes, Eugene, OR) following the manufacturer instructions with slight modifications. Briefly, 1×10^5 bone marrow derived cells were plated and differentiated into macrophages as previously described. Macrophages were treated with 200 μ g/mL of ECM or cytokine controls for 18 h at 37 $^{\circ}$ C, 5% CO $_2$. After treatment, macrophages were washed with PBS and incubated with 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide) solution for 2 h. The straight-line equation from the standard curve was used to interpolate the concentration of cells reducing the MTT after the exposure to the ECM digests. As 1×10^5 cells were initially seeded, the value is presented as the percentage of reducing cells relative to the initial value (1×10^5 cells).

The formazan produced by reduction of the MTT was diluted with 50 μ L of dimethyl sulfoxide (DMSO) and its concentration determined by optical density at 540 nm. The metabolic activity of macrophages was calculated from a standard curve. Results were presented relative to untreated (MCSF only) macrophages.

Phagocytosis

The ability of macrophages to phagocytose fluorescent latex microspheres was evaluated as previously described⁴⁴ with some modifications. After treatment of 1×10^6 bone marrow-derived macrophages with 200 μ g/mL of ECM or cytokine controls for 18 h, macrophages were rinsed with PBS and incubated with 4.55×10^7 particles/mL of Fluoresbrite YG Microspheres 1.00 μ m (Polysciences, Warrington, PA) in complete medium for 15 min at 37 $^{\circ}$ C, 5% CO $_2$. After incubation with microparticles, macrophages were washed with PBS and harvested with Accutase $^{\circ}$ solution. Cells were centrifuged and rinsed with PBS followed by a counterstain with viability dye eFluor 780 (eBioscience, San Diego, CA) at a dilution of 1:1000. A non-phagocytic cell line, C2C12 mouse myoblasts, was used as a negative control. The percentage of phagocytic macrophages was determined by flow cytometry.

Antimicrobial activity

ECM bioscaffolds from each tissue-type were exposed to proliferating *Staphylococcus aureus* (*S. aureus*) bacteria for evaluation of antimicrobial activity as previously described.⁴⁵ Briefly, an isolated colony of *S. aureus* (American Type Culture Collection 29213, clinical isolate) grown on tryptic soy agar was used to inoculate 10 mL of tryptic soy broth. The bacteria were expanded in suspension overnight on a rotary shaker at 37 $^{\circ}$ C. The bacteria were then diluted to 5×10^5 CFU/mL,⁴⁶ and 150 mL of bacterial suspension were added to each well of a 96-well microplate; ECM was added at a concentration of 200 μ g/mL to the bacterial suspension. Secreted products of ECM-treated macrophages were derived using the following method: macrophages were treated for 18 h with solubilized ECM or cytokine controls as described. After 18 h, cells were washed with PBS and medium was replaced with serum free, antibiotic free, ECM-free medium for 5 h, after which time the medium was collected and was diluted at a 1:1 ratio with broth. Samples tested included solubilized ECM from each tissue type, a negative control of medium alone, and pepsin as a carrier control. Each sample was tested in triplicate. The bacterial growth in each well was monitored over the course of 24 h using absorbance readings at 570 nm with a BioRad 680 microplate reader.

Statistical analysis

A one-way ANOVA was used for all comparisons between groups with an LSD post hoc analysis. All statistical analysis

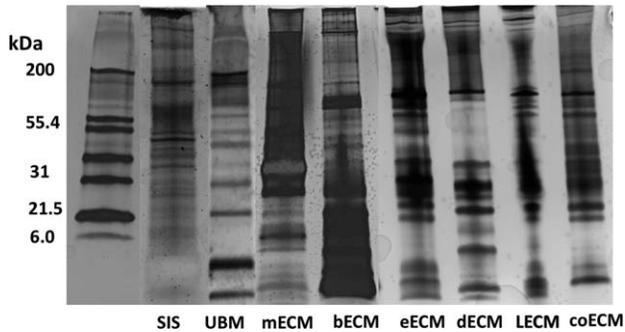


FIGURE 1. SDS PAGE gel analysis of ECM degradation products. Degradation products of ECM bioscaffolds derived from different source tissues were separated using SDS PAGE gel electrophoresis and show distinct banding patterns. (SIS = small intestinal submucosa, UBM = urinary bladder matrix, mECM = skeletal muscle ECM, bECM = brain ECM, eECM = esophageal ECM, dECM = dermal ECM, LECM = liver ECM, coECM = colonic ECM).

used SPSS Statistical Analysis Software (SPSS, IBM, Chicago, IL). Error bars represent standard deviation.

RESULTS

ECM bioscaffolds derived from different source tissues have distinct compositions

SDS PAGE gel analysis of solubilized ECM bioscaffolds show distinct banding patterns following silver stain, indicating that ECM bioscaffolds derived from different source tissues have distinct compositions (Fig. 1).

ECM differentially affect macrophage surface marker expression

Immunolabeling for indicators of the M1 or M2 phenotype using iNOS and Fizz1, respectively, shows that ECM bioscaffolds derived from different source tissues promote different expression patterns [(Fig. 2(A)]. Specifically, SIS-ECM, UBM, bECM, eECM, and coECM promote a predominant Fizz1+ (M2-like) macrophage phenotype with minimal iNOS expression [Fig. 2(B)]. Conversely, dECM shows a predominant iNOS+ (M1-like) phenotype [Fig. 2(B)]. mECM and LECM do not show significant increases in iNOS or Fizz1 expression when compared with untreated controls [Fig. 2(B)].

ECM differentially increase M1-like and M2-like macrophage protein expression

Western blotting shows that SIS, UBM, eECM, and coECM treated macrophages significantly increased CD206 expression similarly to the IL-4 treated control [Fig. 3(B)]. IFN γ /LPS treatment as well as mECM, bECM, dECM, and LECM are characterized by decreased CD206 expression [Fig. 3(B)]. SIS, bECM, and eECM are characterized by a significant decrease of macrophage iNOS expression when compared to the IFN γ /LPS treated control; whereas UBM, mECM, dECM, LECM, and coECM do not decrease macrophage iNOS expression [Fig. 3(D)].

Exposure to ECM differentially affects macrophage viability

ECM bioscaffolds differentially affect macrophage viability. At the evaluated concentration, none of the tissue-type ECMs cause a decrease in cell viability of >20%. Macrophages exposed to eECM (84.14%), LECM (83.34%), and bECM (83.28%) showed the lowest cell viability with significant differences when compared with non-activated M0 macrophages (93.74%) (Fig. 4).

Exposure to ECM differentially affects macrophage metabolism

Exposure of macrophages to tissue-specific ECM differentially modified the macrophage metabolic activity. SIS-ECM, eECM, and LECM maintain cell metabolism activity when compared to untreated macrophages and cytokine-treated macrophages. In contrast, mECM, dECM, coECM, and bECM decrease macrophage MTT metabolism by >50%. UBM treatment also resulted in a significant decrease in MTT metabolic activity when compared to the untreated control (Fig. 5).

Phagocytic capability of macrophages does not significantly differ with phenotype

Phagocytic capability of macrophages was unaffected by treatment with ECM and/or cytokine controls (Fig. 6).

ECM treated macrophages exert antimicrobial effects

Secreted products from cytokine-treated and ECM-treated macrophages show an increased antimicrobial effect when compared to pepsin-treated and untreated controls. ECM activates macrophages similarly to cytokine-treated macrophages with respect to antimicrobial activity (Fig. 7).

DISCUSSION

Biologic scaffold materials composed of mammalian extracellular matrix (ECM) have been associated with favorable preclinical and clinical remodeling outcomes when used as a therapeutic approach following tissue damage or disease.^{15,16,47-49} Xenogeneic ECM bioscaffolds that are thoroughly decellularized and relatively free of cell remnants are typically associated with robust biologic activity including the ability to recruit endogenous stem/progenitor cells and modulate the host innate immune response to injury.⁵⁰ It has previously been established that ECM is able to promote a shift from the default wound healing response to injury (i.e., fibrous scar tissue formation) toward constructive (i.e., functional and site-appropriate) tissue remodeling.⁵¹ Though the mechanisms responsible for this response are only partially understood, one important and necessary event is an early transition in responding macrophage phenotype; specifically from an M1-like, pro-inflammatory phenotype to an M2-like, regulatory and pro-remodeling phenotype following scaffold implantation and subsequent degradation within host tissue.^{3,4,23}

The extent of ECM-mediated constructive remodeling can differ depending upon a number of variables involved in ECM bioscaffold preparation including source animal

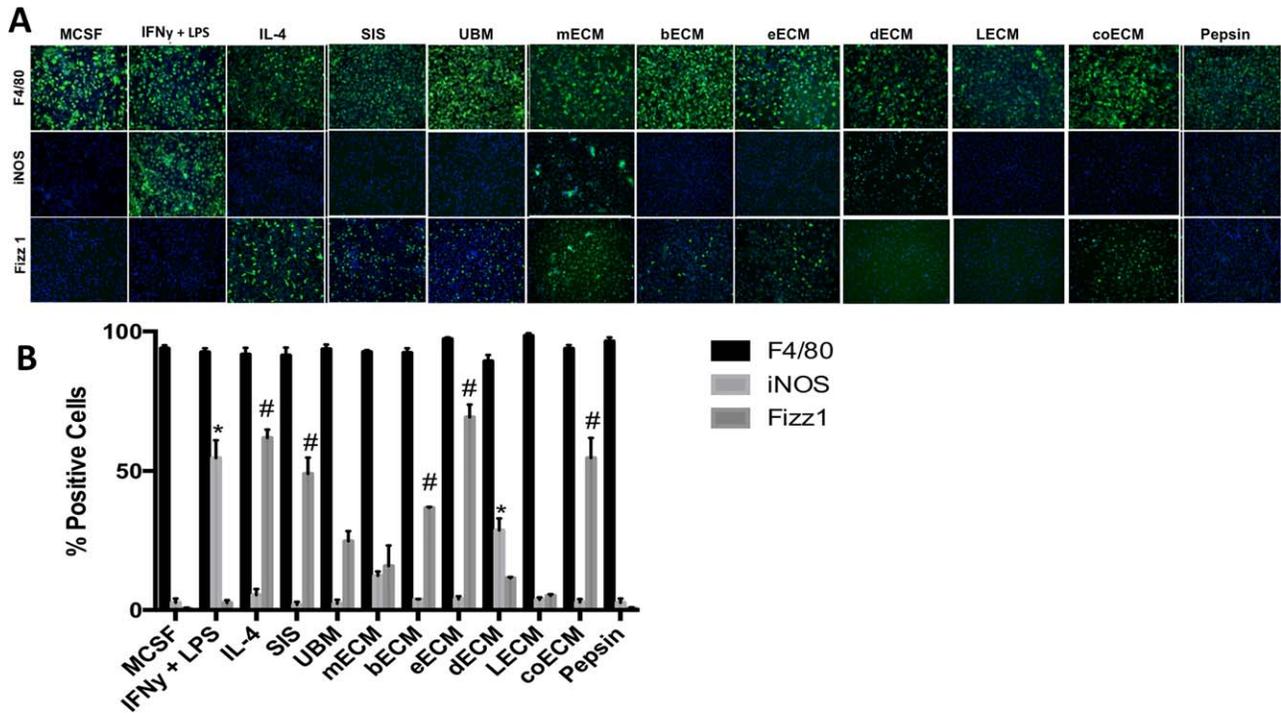


FIGURE 2. Immunolabeling of ECM treated macrophages. A: Macrophages were fixed with 2% paraformaldehyde following 18 h of treatment with cytokines or ECM degradation products and immunolabeled for indicators of the M1 or M2 phenotypes (iNOS, Fizz1, respectively). F4/80 was used as a pan macrophage marker. B: Results were quantified using CellProfiler Image analysis software and show that SIS, bECM, eECM, and coECM promote a predominant M2-like macrophage phenotype, whereas dECM promotes a predominant M1-like macrophage phenotype. (MCSF = macrophage colony stimulating factor, SIS = small intestinal submucosa, UBM = urinary bladder matrix, mECM = skeletal muscle ECM, bECM = brain ECM, eECM = esophageal ECM, dECM = dermal ECM, LECM = liver ECM, coECM = colonic ECM). (* and # indicate $p < 0.05$ when compared to MCSF group for iNOS and Fizz1 quantification, respectively. $n = 8$. Error bars represent standard error of the mean. Light exposure times were standardized to a negative isotype control and kept constant across images).

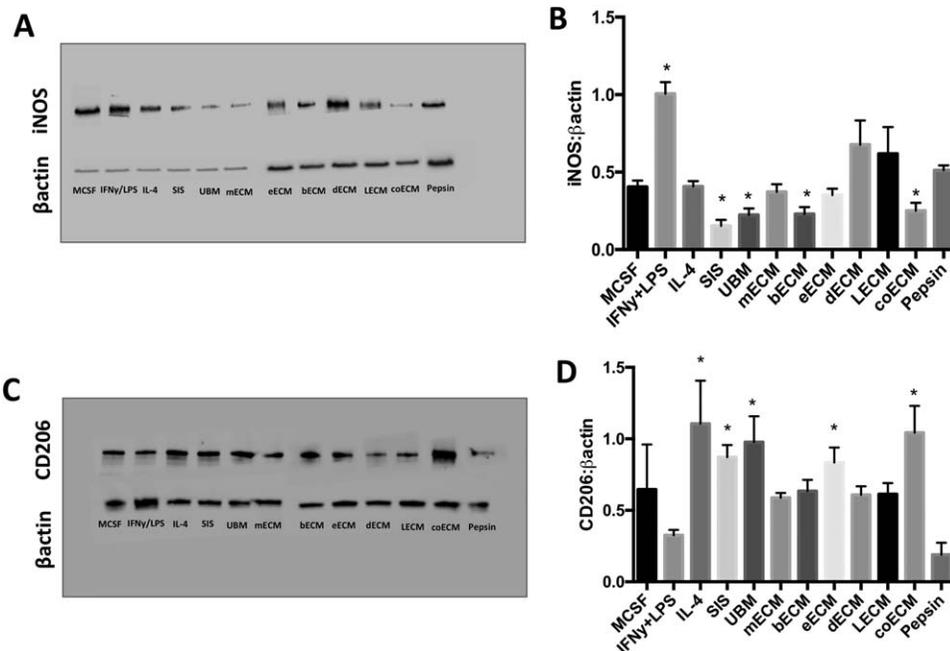


FIGURE 3. Western blotting of ECM-treated macrophages. A: Macrophage lysates were collected and probed for the presence of iNOS and (C) CD206 as M1 and M2-like protein markers, respectively. B: Treatment with SIS, UBM, bECM, and coECM promotes a significant decrease in iNOS expression when compared to the vehicle (pepsin) control treatment. D: Treatment with SIS, UBM, eECM, and coECM promotes an increase in CD206 expression similarly to IL-4 treated macrophages when compared to pepsin treated macrophages. (MCSF = macrophage colony stimulating factor, SIS = small intestinal submucosa, UBM = urinary bladder matrix, mECM = skeletal muscle ECM, bECM = brain ECM, eECM = esophageal ECM, dECM = dermal ECM, LECM = liver ECM, coECM = colonic ECM, * indicates $p < 0.05$ compared to the vehicle control treatment, error bars represent standard error of the mean, $n = 6$).

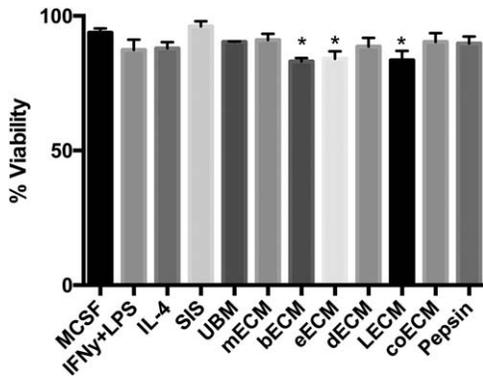


FIGURE 4. Macrophage viability analysis. The viability of macrophages following treatment with cytokines or ECM degradation products was analyzed using trypan blue. Macrophage viability significantly decreases with eECM, LECM, and bECM treatment. (MCSF = macrophage colony stimulating factor, SIS = small intestinal submucosa, UBM = urinary bladder matrix, mECM = skeletal muscle ECM, bECM = brain ECM, eECM = esophageal ECM, dECM = dermal ECM, LECM = liver ECM, coECM = colonic ECM, * indicates $p < 0.05$ when compared to the untreated control, error bars represent standard deviation, $n = 3$).

age,⁵² use of chemical crosslinking agents,⁴ storage conditions,²⁸ extent of decellularization,⁵⁰ terminal sterilization methods,⁵³ and the tissue from which the ECM was derived,^{54,55} among others. ECM bioscaffolds have been shown to direct endogenous cell behavior and influence the local tissue microenvironment. The availability of these bioactive molecules to surrounding host tissue/cells is dependent upon the degradation of ECM bioscaffolds following implantation, subsequently releasing and/or exposing matrix cryptic peptide sites that have been shown to be

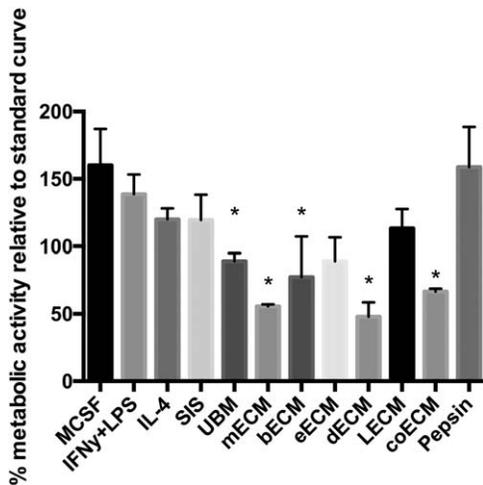


FIGURE 5. MTT Metabolism of Macrophages. MTT analysis shows treatment with mECM, dECM, coECM, or bECM reduces metabolic activity of macrophages when compared to the untreated control. UBM significantly increases MTT metabolism when compared to untreated macrophages, whereas mECM, bECM, dECM, and coECM result in a significant decrease. (MCSF = macrophage colony stimulating factor, SIS = small intestinal submucosa, UBM = urinary bladder matrix, mECM = skeletal muscle ECM, bECM = brain ECM, eECM = esophageal ECM, dECM = dermal ECM, LECM = liver ECM, coECM = colonic ECM, * indicates $p < 0.05$ when compared to untreated macrophages, error bars represent standard deviation).

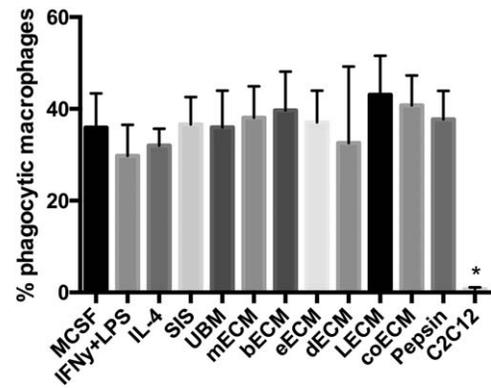


FIGURE 6. Phagocytic capacity of macrophages. Fluorophore-conjugated bioparticle uptake was used as a measure of phagocytic activity of macrophages. Treatment with cytokines or ECM degradation products did not significantly change phagocytosis (MCSF = macrophage colony stimulating factor, SIS = small intestinal submucosa, UBM = urinary bladder matrix, mECM = skeletal muscle ECM, bECM = brain ECM, eECM = esophageal ECM, dECM = dermal ECM, LECM = liver ECM, coECM = colonic ECM, * indicates $p < 0.05$ when compared to untreated macrophages, error bars represent standard deviation, $n = 3$).

chemotactic and mitogenic^{56,57} for progenitor cells and able to induce their differentiation.²⁶ It is logical to assume that ECM derived from homologous source tissue (i.e., from the same tissue that is to be replaced) would contain the inherent structural and biochemical milieu required for tissue-specific differentiation and would represent the optimal environment for such a tissue's associated cells. Some studies have shown that homologous ECM is preferable and maintains tissue specific cell phenotypes.^{54,55,58-61} However, other studies show that heterologous ECM is adequate in promoting site-appropriate tissue deposition.^{16,33} Whether there are differences in the ability of ECM bioscaffolds

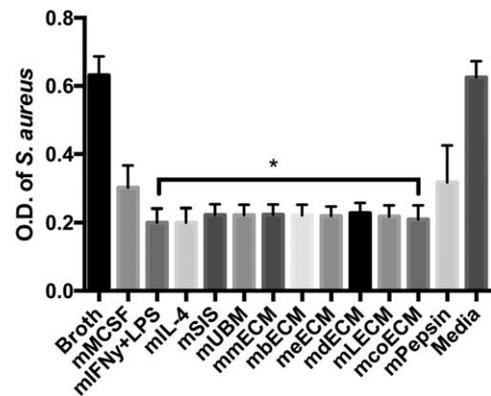


FIGURE 7. Indirect antimicrobial activity of ECM degradation products. *S. aureus* growth was used to determine the antimicrobial effects of macrophages exposed to ECM degradation products. After 18 hours, secreted products from ECM-treated macrophages significantly inhibit *S. aureus* growth, similarly to cytokine-treated macrophages, when compared to untreated macrophages and the negative control (broth). (MCSF = macrophage colony stimulating factor, SIS = small intestinal submucosa, UBM = urinary bladder matrix, mECM = skeletal muscle ECM, bECM = brain ECM, eECM = esophageal ECM, dECM = dermal ECM, LECM = liver ECM, coECM = colonic ECM, * indicates $p < 0.05$ when compared to broth, error bars represent standard deviation, $n = 4$).

derived from different source tissues to directly influence macrophage phenotype has not been previously evaluated. The present study shows that the source tissue from which an ECM bioscaffold is derived can indeed be a determining factor with respect to the macrophage response. While most ECM bioscaffolds promote an M2-like phenotype *in vitro*, surface marker expression shows some exceptions including skeletal muscle ECM (mECM), dermal ECM (dECM), and liver ECM (LECM) treated macrophages which show lower M2-like Fizz1 and CD206 expression with higher levels of M1-like iNOS expression.

It is plausible that these differences in phenotype could be a result of the preparation methods, specifically the method of decellularization utilized for different tissue types. In the present study, all ECMs were decellularized in accordance with previously established protocols designed to meet recognized minimum criteria for decellularization (i.e., no visible intact nuclei by hematoxylin and eosin staining, remnant DNA concentration <50 ng/mg dry weight, and DNA fragment length <200 basepairs).³⁵ For example, SIS-ECM and UBM are prepared with relatively mild decellularization methods whereas skeletal muscle ECM or esophageal ECM are exposed to a series of enzymatic, detergent, and chemical treatments. Variations of decellularization protocols likely contribute to the distinct protein profile for each tissue ECM as shown in the SDS PAGE gel analysis. A proteomic analysis has been conducted using solubilized urinary bladder matrix with hundreds of proteins identified within the solubilized scaffold.⁶² However, it should be noted that proteomic analysis is a function of the particular solubilization process, which in itself generates even more proteins. The present study shows that while there are differences in protein content between each ECM bioscaffold type, it would be difficult to determine which of these differences contribute to any differences in bioactivity, let alone macrophage phenotype specifically due to the shear number and overlap of proteins within the different scaffolds. Previous studies have shown that the macrophage response differs when exposed to fractions of structural and soluble components of the ECM.⁶³ It is likely that decellularization protocols also impact the relative constituents of solubilized ECM utilized in the present study. However, whether a specific peptide or combinations of peptides is responsible for a phenotypic change in macrophages is unknown and warrants future study. The presence of residual detergent could also be a contributing factor in the differences in macrophage responses to different ECM bioscaffolds. Variation in detergents used for tissue decellularization has been shown to have an impact upon the basement membrane complex of urinary bladder matrix, specifically the extent of collagen denaturation, glycosaminoglycan (GAG) concentration, and cellular infiltration, growth, and differentiation.⁶⁴ It is likely that residual detergent remains within the pepsin-solubilized scaffolds used in the present study, and could have an impact upon the macrophage response. However, the objective of the present work was to compare the effects of ECM bioscaffolds and not decellularization methods, though the question of the

impact of specific detergents upon the macrophage response warrants investigation.

A plausible rationale for selecting ECM bioscaffolds derived from one tissue source over another for a given application could include the ability to influence macrophage phenotype. Interestingly, gastrointestinal-derived ECM analyzed in the present study (SIS-ECM, eECM, coECM) promotes a heightened M2-like protein expression profile and diminished M1-like protein expression profile. Moreover, it has been shown that resident macrophages within the gastrointestinal tract retain a more immunotolerant (i.e., more M2-like) phenotype. Perhaps macrophage phenotype is partially determined by the native ECM within the gastrointestinal tract.

In general, the present study shows that regardless of the source tissue from which it is derived, ECM stimulated macrophages show a distinct phenotype when compared to the canonically activated IFN γ /LPS and IL-4 treated macrophages. In general, ECM induces a shift toward an M2-like phenotype. No significant differences were seen in macrophage phagocytosis. Metabolic activity of ECM treated macrophages, in the case of treatment with mECM, dECM, coECM, or bECM, is lowered. The results herein suggest that this decreased metabolic activity is not generated by loss of cell viability (i.e., apoptosis and necrosis), as corroborated by trypan blue exclusion assay results, but by changes in redox states. In this sense, it has been previously shown that cellular metabolism is differentially regulated in macrophage activation to meet the energetic needs of each phenotype in the local microenvironment,^{65,66} with M1-like macrophages requiring greater amounts of NADH (increased redox potential) than its counterpart M2-like phenotype.^{67,68} Since MTT assay, widely used to determine cell viability, incorporates the reduction of MTT to formazan in a NADH-dependent mechanism mainly outside the mitochondria,⁶⁸ this technique could be used as an indirect first approximation to determine the metabolic changes in macrophages. However, additional studies are required to identify the mechanisms involved in the metabolic activation of the macrophages exposed to tissue-specific ECM.

ECM treated macrophages show similar antimicrobial activity to both IFN γ /LPS and IL-4 treated macrophages. When compared to untreated or pepsin-treated macrophages, cytokine or ECM activated macrophages promoted a more potent antimicrobial effect, though no significant differences were found when comparing the effects from macrophages treated with different ECM bioscaffolds. These results show that ECM can indirectly contribute to antimicrobial activity through macrophage activation. Such results also highlight the heterogeneity of macrophages and emphasize the need to evaluate these cells using multiple metrics for comprehensive characterization.

The present study has several limitations. Although macrophages are a key player in tissue-mediated remodeling across species, only mouse bone marrow derived macrophages were utilized for phenotypic characterization following ECM exposure. Whether these same trends will be corroborated utilizing human macrophages should be

investigated. Additionally, the present study did not investigate the specific effects of individual decellularization methods upon an ECM's ability to influence macrophage phenotype. It is likely that a different decellularization protocol, one that does not sufficiently lower DNA content as those used in the present study or otherwise modifies the molecular profile of the ECM, would influence macrophage phenotype.

CONCLUSION

The results herein show that ECM is able to induce changes in macrophage phenotype and function. Overall, ECM promotes a macrophage phenotype that is distinct from that of cytokine-activated macrophages. The direct effects of ECM bioscaffolds upon macrophage phenotype could have implications for the use of site-specific ECM in therapeutic applications. The findings reported show the heterogeneity of macrophages and the differences in bioactive molecules generated from ECM derived from diverse source tissues.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Lynda Guzik for her assistance with flow cytometry analysis. Jenna Dziki was partially supported by the National Science Foundation Graduate Student Research Fellowship.

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