Macrophage Phenotype in Response to ECM Bioscaffolds

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Abstract

Macrophage presence and phenotype are critical determinants of the healing response following injury. Downregulation of the pro-inflammatory macrophage phenotype has been associated with the therapeutic use of bioscaffolds composed of extracellular matrix (ECM), but phenotypic characterization of macrophages has typically been limited to small number of non-specific cell surface markers or expressed proteins. The present study determined the response of both primary murine bone marrow derived macrophages (BMDM) and a transformed human mononuclear cell line (THP-1 cells) to degradation products of two different, commonly used ECM bioscaffolds; urinary bladder matrix (UBM-ECM) and small intestinal submucosa (SIS-ECM). Quantified cell responses included gene expression, protein expression, commonly used cell surface markers, and functional assays. Results showed that the phenotype elicited by ECM exposure (M_{ECM}) is distinct from both the classically activated IFNγ + LPS phenotype and the alternatively activated IL-4 phenotype. Furthermore, the BMDM and THP-1 macrophages responded differently to identical stimuli, and UBM-ECM and SIS-ECM bioscaffolds induced similar, yet distinct phenotypic profiles. The results of this study not only characterized an M_{ECM} phenotype that has anti-inflammatory traits but also showed the risks and challenges of making conclusions about the role of macrophage mediated events without consideration of the source of macrophages and the limitations of individual cell markers.

Keywords: ECM (extracellular matrix); macrophages; activation; THP-1; BMDM; phenotype.

1. Introduction:

Biologic scaffold materials composed of extracellular matrix (ECM) have been used in both preclinical and clinical studies to facilitate the functional reconstruction of soft tissues including the esophagus [1], skeletal muscle [2], and myocardium [3], among others [4-6]. Results of such studies have varied from excellent to unacceptable [7], and the reasons for disparate results have been attributed to variables such as the methods used to decellularize source tissues [8, 9], the use of chemical crosslinking agents that inhibit scaffold degradation [10], and other factors [11, 12]. Arguably the most important mechanism by which ECM bioscaffolds influence tissue remodeling and functional outcome is the modulation of macrophage phenotype [13]. In fact, the ratio of M2-like/M_{IL-4} (regulatory/anti-inflammatory) to M1-like/$M_{IFN\gamma+LPS}$ (pro-inflammatory) macrophages has been shown to be a predictor of favorable outcomes in multiple studies [14-17].

Macrophages have long been recognized as phagocytes with pro-inflammatory and cytotoxic functions. However, it is now understood that these cells also play essential roles in the resolution of inflammation [18, 19], normal tissue development [20], and in blastemal-based epimorphic regeneration in species such as the axolotyl [21]. These "non-classical" macrophage activities are increasingly tied to shifts in the balance of M1:M2 macrophages participating in the host inflammatory reaction. While the description of macrophages as having an M1 or M2 phenotype is operationally simple and facilitates discussion, supra-physiologic amounts of signaling molecules such as cytokines, toll-like receptor agonists, and growth factors have been used *in-vitro* to induce these extremes of pro-inflammatory or anti-inflammatory phenotype [22-

24]. However, such conditions do not mimic the *in-vivo* complexity of macrophage activation. In fact, virtually any stimulus will likely elicit a macrophage phenotype that exists somewhere between the extremes.

The macrophage response to biomaterials is a critical predictor of downstream success or failure with respect to clinical outcomes. Though there is now widespread recognition of the heterogeneity and plasticity of macrophage phenotype, most studies evaluating / describing the macrophage response to a biomaterial have included only single-marker methods followed by conclusive statements regarding cell phenotype. The present manuscript reviews the macrophage response to biologic scaffold materials, particularly those derived from mammalian extracellular matrix.

Given the diversity and broad scope of endogenous signaling molecules resident within extracellular matrix (e.g., growth factors, cytokines, cryptic peptides and miRNA), and the widespread clinical use of ECM bioscaffolds in tissue reconstruction, the "M_{ecm}" phenotype is characterized in the present study. Recognizing the limitations of only a single cell property as a defining identifier of macrophage phenotype, several parameters are evaluated including transcription factor profile, gene expression, protein expression, cell surface markers and functional properties. ECM bioscaffolds derived from two separate tissue sources (porcine small intestine and urinary bladder) are used to activate two macrophage populations that are commonly used for such studies: primary mouse bone marrow derived macrophages and THP-1 cells (a human mononuclear cell line). To further evaluate macrophage responses to ECM signaling molecules, the activation state of the cells at the time of ECM stimulation is considered.

Specifically, both naive macrophages and macrophages that have been activated with IFNγ+LPS are used in the present study.

2. Results

The terminology used to describe various states of macrophage activation (often referred to as "polarization") has contributed to potentially misleading conclusions regarding the role of macrophages in various physiologic and pathologic processes. For example, macrophages have been identified as M1 (pro-inflammatory) or M2 (anti-inflammatory), or given labels such as "regulatory" based upon selected surface markers or associated effector molecules. Recommendations for standard nomenclature based upon definition of the activator were published in 2014 [25], and this terminology will be used herein whenever possible (e.g., $M_{IFN\gamma+LPS}$ and M_{IL-4}). Macrophages stimulated with solubilized extracellular matrix (ECM) will be designated as M_{ECM} ($M_{UBM-ECM}$ and $M_{SIS-ECM}$).

2.1 M_{ECM} has a distinct gene expression profile

Human THP-1 monocytes and mouse bone marrow (BMDM) were differentiated into macrophages to generate an M∅ phenotype. M∅ macrophages were then treated for 24h with IFNγ+LPS to establish an $M_{IFN\gamma+LPS}$ phenotype, IL-4 to establish an M_{IL-4} phenotype, or either UBM-ECM or SIS-ECM to establish an M_{ECM} ($M_{UBM-ECM}$ / $M_{SIS-ECM}$) phenotype. In a separate experiment, M∅ macrophages were challenged with IFNγ+LPS for 6 hours followed by a 24 hour treatment with UBM or SIS. Downstream analyses included: 1) Gene expression of 30 commonly investigated macrophage activation markers, surface markers, cytokines, transcription factors and metabolic

markers; 2) Protein expression of the most highly regulated markers; and 3) macrophage function as assessed by phagocytosis and nitric oxide production (Supplementary figure1).

Thirty commonly used macrophage markers of activation, including surface markers, cytokines, transcription factors, and metabolic markers were analyzed by qPCR to better understand the gene expression signature of treated macrophages. Gene expression data are displayed as a heatmap in Figure 1. Clear differences between the gene expression signature of THP-1 macrophages and BMDM are shown. Exposure of THP-1 macrophages to IFNγ+LPS resulted in an increase in almost the entire gene panel, while changes following exposure to IL-4 were relatively mild. In contrast, exposure to either UBM-ECM or SIS-ECM degradation products resulted in only minor changes in gene expression (Figure 1A).

Exposure of BMDM to IFNγ+LPS or IL-4 led to substantial changes in gene expression with contrasting profiles. The gene expression profile generated by exposure to UBM-ECM differed from that generated by exposure to SIS-ECM but there were areas of overlap. Notably, IFNγ+LPS and UBM-ECM treated BMDMs have a similar gene expression profile (Figure 1B).

When THP-1 macrophages were challenged with IFNγ+LPS for 6 hours followed by exposure to UBM-ECM or SIS-ECM for an additional 24 hours, no major changes in gene expression were observed (Figure 1A). However, there was a significant change in the gene expression signature in BMDM (Figure 1B). In addition to the different response between the two macrophage populations, there were also clear differences between UBM-ECM and SIS-ECM treatment groups post-cytokine treatment. Both UBM-ECM and SIS-ECM treatment groups showed a decrease in the transcription factor gene expression cluster, particularly when the cells were pre-treated with

IFNγ+LPS. Gene expression values were normalized to those of untreated macrophages (M∅). IFNγ+LPS activated macrophages were normalized to IFNγ+LPS followed by media treatment.

Principal component analysis (PCA) was conducted to identify the dominant members of the transcriptional signature associated with the test groups for further evaluation of downstream protein expression. BMDM and THP-1 macrophages treated for 24 hours with cytokines or ECM degradation products were scored and visually clustered by PCA. For THP-1 macrophages, CD206, KLF4 and TGM2 were found to be the genes most associated with IL-4 activation, and TNFα, STAT1 and IRF3 were associated with IFNγ+LPS activation (Figure 1C). The BMDM showed Fizz-1, KLF-4 and Arg1 as the most differentially expressed genes associated with IL-4 activation, and TNFα, STAT1 and iNOS were the most highly regulated genes with IFNγ+LPS activation (Figure 1D). Genes that are commonly cited in the literature as macrophage activation markers, and the genes identified in PCA data output, were chosen for further downstream protein analyses.

2.2 ECM degradation products and IL-4 promote similar protein expression profile

Protein expression was evaluated by western blot analysis and immunofluorescent labeling. THP-1 macrophages activated for 24 hours with UBM-ECM or SIS-ECM induced TGM2 and CD206 (M$_{IL-4}$ associated markers). However, no changes were noted in the M$_{IFNγ+LPS}$ associated marker TNFα and only a mild change in iNOS expression with SIS-ECM activation. CD11b was used as a pan macrophage control marker for THP-1 macrophages (Figure 2A). Macrophages activated with IFNγ+LPS followed by exposure to ECM degradation products showed a similar trend to the 24 hours treatment groups. When THP-1 macrophages were exposed to either UBM-ECM or SIS-ECM following activation with INFγ+LPS, both ECMs induced TGM2 and CD206 positive cells.

UBM-ECM and SIS-ECM both caused a reduction in iNOS expression by THP-1 macrophages that had been activated with IFNγ+ LPS. However, both UBM-ECM and SIS-ECM induced TNFα positive cells when macrophages were activated with IFNγ+LPS (Figure 2A).

BMDM show Fizz-1 and Arg1 (M_{IL-4} associated markers in mice) expression after activation with UBM-ECM and SIS-ECM for 24 hours. In addition, BMDM were positive for the $M_{IFN\gamma+LPS}$ associated marker TNFα after exposure to ECM degradation products, but were not positive for iNOS. F4/80 was used as a pan macrophage control marker for BMDM (Figure 2C). Similarly, macrophages activated with IFNγ+LPS followed by treatment with ECM degradation products showed enhanced Fizz-1 expression, but not enhanced Arg1 expression. Interestingly, both UBM-ECM and SIS-ECM inhibited iNOS expression and enhanced TNFα expression after pre-activation with IFNγ+LPS.

Western blot analysis was used to determine relative protein expression of the specified genes that showed the greatest change in activity in response to specific treatments. For the $M_{IFN\gamma+LPS}$ associated markers, the THP-1 macrophages activated for 24 hours with UBM-ECM or SIS-ECM showed that the amount of STAT1 was comparable to that of M$\varnothing$ and M_{IL-4} cells, and significantly lower than that of the $M_{IFN\gamma+LPS}$ cells. No changes were noted in TNFα and IRF3 (Figure 2B).

For the M_{IL-4} associated markers, TGM2 and KL4 protein expression were increased after IL-4 activation. No significant changes were found between the M_{ECM} groups and the M$\varnothing$ and $M_{IFN\gamma+LPS}$ phenotypes. When macrophages were first activated IFNγ+LPS, followed by exposure to SIS-ECM, a decrease in KLF4 protein expression was noted (Figure 2B).

BMDM exposed to UBM-ECM or SIS-ECM degradation products show similar findings to THP-1 macrophages with some small differences. For example, BMDM activated by 24h exposure to UBM-ECM showed increased expression of STAT1 to a level similar to that of the $M_{IFN\gamma+LPS}$ treatment group, which was not seen in the THP-1 groups. However, in both populations of macrophages, no changes were noted in TNFα expression levels (Figure 2D). Notably, an increase in iNOS expression was found only in the BMDM $M_{IFN\gamma+LPS}$ group. When macrophages were activated with IFNγ+LPS followed by UBM or SIS treatment, no changes were noted in iNOS and TNFα expression levels when compared to media controls. However, SIS-ECM treatment inhibited STAT1 protein expression for the group first activated by IFNγ+LPS. For the M_{IL-4} associated markers, M_{IL-4} significantly increased Arg1 protein expression, and SIS-ECM significantly increased Fizz-1 expression. No changes were noted in KLF4 (Figure 2D). β-actin was used as a loading control and the colorimetric intensity of the bands for each treatment group was standardized to its respective β-actin band intensity. Quantification of immunolabeling images using CellProfiler software supported the qualitative interpretation of the results (Supplementary figure 2A-B). Densitometry evaluation of each blot can be found in Supplementary figure 3A-D.

2.3 ECM degradation products affect macrophage functional activity

THP1 macrophages showed low levels of phagocytosis across all tested conditions. Cytokine treatment did not significantly enhance the phagocytic function of THP1 macrophages. However, UBM-ECM activation alone caused an increase in THP1 phagocytosis (Figure 3A). BMDM showed measurable basal phagocytic function. Phagocytosis by BMDM increased with IFNγ+LPS and no

notable changes were detected following IL-4 activation (Figure 3B). Similar to the THP-1 macrophages, UBM-ECM activation resulted in an increase in phagocytosis. In both BMDM and THp1 macrophages, activation with IFNγ+LPS for 6h prior to 24h exposure to UBM-ECM or SIS-ECM did not affect the cells' phagocytic ability. THP1 macrophages did not produce nitric oxide (NO) in response to IFNγ+LPS or IL-4 stimulus. However, UBM treatment did show a slight increase in NO production. Interestingly, THP-1 macrophages challenged with IFNγ+LPS followed by UBM-ECM exposure did show a significant increase in NO production, but such changes were not observed with SIS-ECM exposure (Figure 3C). In BMDM, NO production increased following IFNγ+LPS. BMDM exposed to SIS or UBM alone had a slight increase in NO production. IFNγ+LPS activation followed by either UBM-ECM or SIS-ECM enhanced or prolonged cytokine effects compared to media controls (Figure 3D).

3. Material and Methods

The present study determined macrophage phenotype following exposure to degradation products derived from ECM bioscaffolds. Two macrophage populations commonly used in in-vitro studies examined: human THP-1 monocytes [American Type Culture Collection (ATCC)] and murine bone marrow derived macrophages. A comprehensive characterization of macrophage cell surface markers, gene expression, protein content, phagocytic capacity, and nitric oxide production was conducted. Based on previous studies, more than 30 different surface markers, transcription factors, cytokines and metabolic markers were selected to evaluate the ECM-induced macrophage phenotype, termed M_{ECM}. In addition, the production of proteins selected based upon PCA analysis was determined by western blotting and immunolabeling. Lastly, macrophage phagocytic activity and nitric oxide production post-

treatment was determined. The variety of methods used to assess the changes in macrophage phenotype are described below.

3.1 Preparation of ECM Bioscaffolds

Porcine urinary bladders from market weight (approximately 110 kg) animals were acquired from Tissue Source, LLC. (Lafayette, Indiana, USA). Urinary bladder matrix (UBM-ECM) was prepared by decellularization using mechanical and chemical methods as previously reported [26]. Briefly, the tunica serosa, tunica muscularis externa, tunica submucosa, and tunica muscularis mucosa were mechanically removed. The luminal urothelial cells of the tunica mucosa were dissociated by washing with sterile water. The remaining tissue consisting of basement membrane and subjacent tunica propria of the tunica mucosa was decellularized by agitation in 0.1% peracetic acid with 4% ethanol for 2 hours at 300 rpm. The tissue was then extensively rinsed with phosphate-buffered saline (PBS) and sterile water. The UBM-ECM was then lyophilized and milled into particulate form using a Wiley Mill with a #60 mesh screen.

Preparation of SIS-ECM has been previously described [27]. Briefly, jejunum was harvested from market weight pigs and split longitudinally. The superficial layers of the tunica mucosa were mechanically removed. Likewise, the tunica serosa and tunica muscularis externa, tunica submucosa, and tunica muscularis mucosa were mechanically removed, leaving the tunica submucosa and basilar portions of the tunica mucosa. Decellularization and disinfection of the tissue occurred by agitation in 0.1% peracetic acid with 4% ethanol for 2 hours at 300 rpm. The tissue was then extensively rinsed with phosphate-buffered saline (PBS) and sterile water. The SIS-ECM was then lyophilized and milled into particulate form using a Wiley Mill with a #60 mesh screen.

3.2 Derivation of ECM Degradation Products

UBM-ECM and SIS-ECM were enzymatically degraded as previously described [28] with pepsin from porcine stomach mucosa (MP Biomedicals) by mixing lyophilized, powdered UBM-ECM (10 mg/mL) and pepsin (1 mg/mL) in 0.01 M HCl (pH 2.0). This solution was stirred at room temperature for 48 hours. After stirring, the UBM slurry was neutralized to a pH of 7.4 in 1× PBS (137 mM NaCl, 2.7 mM KCl, 12 mM Phosphate, Fisher Scientific, Waltham, MA) to inactivate the pepsin.

3.3 Macrophage culture

THP-1 human monocytes (TIB-202™) were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA) and maintained in RPMI, 10% FBS, 1% penicillin/streptomycin, and 50 uM of 2-Mercaptoethanol in a humidified atmosphere at 37 °C with 5% CO2. Two million THP-1 cells were plated with 320 nM phorbol 12-myristate 13-acetate (PMA) to induce differentiation into macrophages. After 24 hours adherent macrophages were washed in PBS and placed in fresh media, followed by 72 hours incubation in fresh media to acquiesce. This protocol has been shown to result in a phenotype that is nearly indistinguishable from human peripheral blood macrophages [28].

Murine bone marrow derived macrophages (BMDM) were isolated as previously described [29]. Briefly, the tibia and femur were isolated from adult, female 6–8-week old C57bl/6 mice obtained from Jackson Laboratories (Bar Harbor, ME). 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, 0.1% beta-mercaptoethanol (Gibco), 100 U/ml penicillin, 100 ug/ml streptomyocin, 10 mM non-essential

amino acids (Gibco), and 10 mM hepes buffer. In a sterile environment, the ends of each bone were transected and the marrow cavity flushed with complete medium using a 30-gauge needle. Harvested cells were washed and plated at 10^6 cell/ml, and allowed to differentiate into macrophages for 7 days at 37 °C, 5% CO2 with complete media changes every 48 h resulting in naïve macrophages.

3.4 Macrophage activation

Macrophages were activated for 24 hours with one of the following: (1) 20 ng/ml IFNγ and 100 ng/ml LPS to promote an $M_{IFN\gamma+LPS}$ phenotype, (2) 20 ng/ml IL-4 to promote an M_{IL-4} phenotype, or [30] 250 ug/ml of UBM-ECM, or SIS-ECM to promote an M_{ECM} phenotype. An equivalent concentration of pepsin was used as control buffer. In a separate group, macrophages were just activated with IFNγ+LPS for 6 hours, as described above, and then exposed to UBM-ECM or SIS-ECM for 24 hours. After the incubation period at 37 °C, cells were washed with sterile PBS and fixed with 2% paraformaldehyde for immunolabeling or harvested with TRIZOL/RIPA buffer for RNA/Protein assessment, respectively. Cells were also assessed for phagocytosis and nitric oxide production.

3.5 RNA isolation and cDNA synthesis

Cellular RNA was isolated using the miRNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcriptase from RNA to cDNA was performed via high capacity RT kit (ABI, Foster City, CA) according to the manufacturer's instructions.

3.6 quantitative polymerase chain reaction (PCR)

Sybr Green gene expression assays (ABI, Foster City, CA) were used to determine the relative expression levels of THP-1: iNOS, TNFα, STAT1, STAT2, STAT5, IRF3, IRF4, IRF5, IL1RN, CD206, TGM2, STAT3, STAT6, KLF4, KLF6, PPARγ. BFKBF3, GLUT1, HIF1a, PGK1, LDHA, HK3, PDK4, RPIA, PPARδ, G6PC3 and PCK2. For BMDM gene expression levels: inos, tnfα, stat1, stat2, stat5, irf3, irf4, irf5, il1rn, cd206, tgm2, stat3, stat6, klf4, klf6, fizz-1, arg1, bfkbf3, glut1, hif1a, hk3, pgk1, pdk4, rpia, ldha, pck1, pck2, g6pc3 and pparδ . Results were analyzed by the ΔΔCt method using β-glucuronidase (β -GUS) control for human, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for mouse, to normalize the results. Fold change was calculated taking untreated as the baseline. Results are displayed in a heat map format created by Java Treeview.

Gene description can be found in Table 1:

Table 1:

Name	Gene Name	Description
ARG1	arginase 1	Arginase catalyzes the hydrolysis of arginine to ornithine and urea. At least two isoforms of mammalian arginase exist (types I and II) which differ in their tissue distribution, subcellular localization, immunologic crossreactivity and physiologic function. Arginase is induced by Th2-type cytokines, which convert arginine into ornithine and subsequently into polyamines and proline [31, 32].
CD206	mannose receptor, C type 1	Mannose receptor C type 1 is a type I membrane receptor that mediates the endocytosis of glycoproteins by macrophages. The recognition of complex carbohydrate structures on glycoproteins is an important part of several biological processes, including cell-cell recognition, serum glycoprotein turnover, and neutralization of pathogens. It has been shown that CD206, the macrophage mannose receptor, is up-regulated following interleukin (IL)-4 stimulation, which led to the advent of the concept of alternative activation of macrophages [31, 33].
Fizz-1	resistin like alpha	Alternatively activated macrophages are characterized by abundant expression of mannose receptor (MR/CD206), CD163, arginase, chitinase-like molecules (Ym-1/2), and resistin-like molecule α (RELMα/Fizz-1) upon stimulation with Th2-type cytokines such as IL-4 and IL-13 [31, 34].

G6PC3	glucose-6-phosphatase catalytic subunit 3	Glucose-6-phosphatase catalytic subunit 3 is the catalytic subunit of glucose-6-phosphatase (G6Pase). In a murine G6PC3 double knockout model of congenital neutropenia syndrome 4, G6PC3 (-/-) macrophages have impaired respiratory bursts, chemotaxis, calcium flux, and phagocytosis as well as lower glucose uptake and low levels of G6P, lactate, and ATP. In addition, trafficking of G6PC3 (-/-) macrophages during an inflammatory response in vivo was impaired [31, 35].
GLUT1	solute carrier family 2 member 1	GLUT1 is a major glucose transporter in the mammalian blood-brain barrier. Glucose is a critical component in the proinflammatory response of macrophages [31, 36].
HIF1α	hypoxia inducible factor 1 alpha subunit	Hypoxia-inducible factor-1 is a heterodimer composed of an alpha and a beta subunit. The HIF1α subunit, like 6-phosphofructo-2-kinase, is involved in hypoxia-mediated glycolytic flux leading to pro-inflammatory macrophage activity. HIF-1α is induced by NFkB and mediates transcription of iNOS and subsequent production of nitric oxide [31, 37].
HK3	hexokinase 3	Hexokinases phosphorylate glucose to produce glucose-6-phosphate, the first step in most glucose metabolism pathways. Studies have shown that HK3 is significantly upregulated in macrophages after treatment with LPS [31, 38].
IL1rα	interleukin 1 receptor antagonist	Interleukin 1 receptor antagonist inhibits the activities of interleukin 1 alpha (IL1A) and beta (IL1B), and modulates a variety of interleukin 1 related immune and inflammatory responses. Studies suggest that IL-1 alpha, IL-3, IL-4 and GM-CSF may play important roles in regulating monocyte IL-1ra production [31, 39].
iNOS	nitric oxide synthase 2	Nitric oxide synthase is expressed in liver and is inducible by a combination of lipopolysaccharide and certain cytokines including IFN-alpha or LPS. NOS2 degrades arginine into OH-arginine and then nitric oxide (NO). NO is a reactive free radical which acts as a biologic mediator in several processes, including neurotransmission and antimicrobial and antitumoral activities [31, 32].
IRF3	interferon regulatory factor 3	Like IRF5, interferon regulatory factor 3 is found in an inactive cytoplasmic form that upon serine/threonine phosphorylation forms a complex with CREBBP. Signaling through the TRIF adaptor pathway activates IRF3, leading to the expression and secretion of type I interferon, such as IFNα and IFNβ [22, 31].
IRF4	interferon regulatory factor 4	Interferon regulatory factor 4 is found in an inactive cytoplasmic form that upon serine/threonine phosphorylation forms a complex with CREBBP. IRF4 has been identified as a key transcription factor that controls M2 macrophage polarization [31, 40].
IRF5	interferon regulatory factor 5	Interferon regulatory factor 5 is found in an inactive state in the cytoplasm. Upon serine/threonine phosphorylation, IRF5 forms a complex with CREBBP. IRF5 acts as a molecular switch influencing whether macrophages promote or inhibit inflammation. IRF5 is required for optimal Akt2 activation, which increases expression of glycolytic pathway genes and HIF1A as well as pro-inflammatory cytokines and M1 polarization [31, 41].

KLF4	Kruppel like factor 4	Kruppel like factor 4 is a zinc finger protein that acts as a transcriptional activator and functions as a tumor suppressor. It has been demonstrated that IL-4-induced macrophage polarization involves induction of STAT6 and Krüppel-like factor 4 (KLF4), which induce each other and promote M2 polarization [31, 42].
KLF6	Kruppel like factor 6	Kruppel like factor 6 is a zinc finger protein that acts as a transcriptional activator and functions as a tumor suppressor. KLF6 has been implicated in the control of macrophage speciation. In human and mouse macrophages, it has been shown that pro-inflammatory (M1-like) stimuli such as LPS and IFN-gamma lead to robust KLF6 expression, while M2-like stimuli such as IL-4 and IL13 suppressed KLF6 [31, 43].
LDHA	lactate dehydrogenase A	Lactate dehydrogenase A catalyzes the conversion of L-lactate and NAD to pyruvate and NADH in the final step of anaerobic glycolysis. HIF-1α induces expression of lactate dehydrogenase, which catalyzes lactate production from pyruvate, thereby limiting the production of acetyl-CoA for the TCA cycle [31, 44].
PCK1	phosphoenolpyruvate carboxykinase 1	Phosphoenolpyruvate carboxykinase 1 is a main control point for the regulation of gluconeogenesis. This cytosolic enzyme, along with GTP, catalyzes the formation of phosphoenolpyruvate from oxaloacetate, with the release of carbon dioxide and GDP. In adipose tissue macrophages derived from obese mice are characterized by enhanced expression of inflammatory cytokines, chemokines and mediators such as TNFα, IL-6, IL-8, CCL2, IL-1β, and iNOS--all factors that suppress insulin signaling and de-repress downstream gluconeogenesis via activation of glucose-6-phosphatase (G6PC) and phosphoenolpyruvate carboxykinase 1 (PCK1) [31, 45].
PCK2	phosphoenolpyruvate carboxykinase 2	Phosphoenolpyruvate carboxykinase 2 is a mitochondrial enzyme that catalyzes the conversion of oxaloacetate to phosphoenolpyruvate in the presence of guanosine triphosphate (GTP). Metabolic modules have been shown to directly influence macrophage polarization [31, 46].
PDK4	pyruvate dehyrogenase kinase 4	Pyruvate dehyrogenase kinase 4 is a member of the PDK/BCKDK protein kinase family. PDK4 is a mitochondrial protein with a histidine kinase domain. It has been shown that strong decreases in the expression of PDK4 leads to an increase in metabolism, particularly the conversion of glucose to acetyl-CoA, thus improving substrate availability for fatty acid synthesis [31, 47].
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	6-phosphofructo-2-kinase belongs to a family of bifunctional proteins that are involved in both the synthesis and degradation of fructose-2,6-bisphosphate, a regulatory molecule that controls glycolysis in eukaryotes. Hypoxia potentiates macrophage glycolytic flux correlating with upregulation of pro-inflammatory activity in a manner that is dependent on 6-phosphofructo-2-kinase [31, 48].
PGK1	phosphoglycerate kinase 1	Phosphoglycerate kinase 1 is a glycolytic enzyme that catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate. Macrophages demonstrate significantly higher expressions of the gene PGK1 under normoxia than monocytes [31, 49].

PPARδ	peroxisome proliferator activated receptor delta	Peroxisome proliferator activated receptor delta is a member of the peroxisome proliferator-activated receptor (PPAR) subfamily of nuclear receptors. PPARs are nuclear hormone receptors that bind peroxisome proliferators and control the size and number of peroxisomes produced by cells. PPARs mediate a variety of biological processes, and may be involved in the development of several chronic diseases, including diabetes, obesity, atherosclerosis, and cancer. Studies have established a role for PPARδ in the regulation of macrophage lipid metabolism and inflammation [31, 50].
PPARγ	peroxisome proliferator activated receptor gamma	Peroxisome proliferator activated receptor gamma is a member of the peroxisome proliferator-activated receptor (PPAR) subfamily of nuclear receptors. PPARs form heterodimers with retinoid X receptors (RXRs) and these heterodimers regulate transcription of various genes. Studies from the Glass and Seed laboratories indicated that pharmacological activation of PPARγ attenuated expression of macrophage inflammatory programs. Studies have established a role for PPARγ in the regulation of macrophage lipid metabolism and inflammation [31, 50].
RPIA	ribose 5-phosphate isomerase A	Ribose 5-phosphate isomerase A is an enzyme that catalyzes the reversible conversion between ribose-5-phosphate and ribulose-5-phosphate in the pentose-phosphate pathway. In granulocyte-macrophage colony stimulating factor (GM-CSF) activated murine bone marrow derived macrophages, RPIA was abundantly expressed in comparison to macrophages activated with macrophage colony stimulating factor (M-CSF). Metabolic processes were significantly different between these two populations with GM-CSF stimulated macrophages showing enhanced glycolytic capability which may correspond with their pro-inflammatory response [31, 51].
STAT1	signal transducer and activator of transcription 1	Upon stimulation with cytokines and growth factors, STAT family members are phosphorylated to form homo- or heterodimers that translocate to the cell nucleus and act as transcription activators. STAT1, in particular, plays a pivotal role in the differentiation/maturation process of monocytes as an early transcription factor initially activated by adherence and then able to modulate the expression of functional genes, such as ICAM-1 and FcgammaRI. IL-4 and IFN-γ, together with TLR stimulation, upregulate SOCS1 (31) and SOCS3 (32), which inhibit STAT1 and STAT3, respectively [22, 31, 52].
STAT2	signal transducer and activator of transcription 2	Upon stimulation with cytokines and growth factors, STAT family members are phosphorylated to form homo- or heterodimers that translocate to the cell nucleus and act as transcription activators. In response to type-1 IFNs (-α and -β), STAT1 and STAT2 are activated altering macrophage polarization [31, 37].
STAT3	signal transducer and activator of transcription 3	Upon stimulation with cytokines and growth factors, STAT family members are phosphorylated to form homo- or heterodimers that translocate to the cell nucleus and act as transcription activators. IL-4 and IFN-γ, together with TLR stimulation, upregulate SOCS1 (31) and SOCS3 (32), which inhibit STAT1 and STAT3, respectively [22, 31].

STAT5	signal transducer and activator of transcription 5	Upon stimulation with cytokines and growth factors, STAT family members are phosphorylated to form homo- or heterodimers that translocate to the cell nucleus and act as transcription activators. STAT5 has been shown to play a role in monocyte activation by LPS. STAT5 is a target for IL-10 and Dex inhibitition of COX-2 expression in activated monocytes [31, 53].
STAT6	signal transducer and activator of transcription 6	Upon stimulation with cytokines and growth factors, STAT family members are phosphorylated to form homo- or heterodimers that translocate to the cell nucleus and act as transcription activators. It has been demonstrated that IL-4-induced macrophage polarization involves induction of STAT6 and Krüppel-like factor 4 (KLF4), which induce each other and promote M2 polarization [31, 42].
TGM2	transglutaminase 2	Transglutaminases are enzymes that catalyze the crosslinking of proteins by epsilon-gamma glutamyl lysine isopeptide bonds. It has been shown that IL-4 activated cells can be characterised by increased expression of TGM2 [31, 54].
TNFα	tumor necrosis factor	Tumor necrosis factor alpha is a proinflammatory cytokine that is mainly secreted by macrophages. Toll-like receptor binding induces the production of TNFα from macrophages that function to activate macrophages [31, 55].

3.7 Macrophage Immunolabeling

To determine macrophage surface marker expression profiles, activated cells were fixed with 2% paraformaldehyde (PFA) for 45 minutes. Primary antibodies used for immunofluorescent labeling on BMDM were: (1) monoclonal anti-F4/80 (Abcam, Cambridge, MA) at 1:200 dilution for a pan-macrophage marker, (2,3) polyclonal anti-iNOS (Abcam, Cambridge, MA) at 1:100 dilution and anti-TNFα (Abcam, Cambridge, MA) at 1:1000 for an M1-like marker, and (4,5) polyclonal anti-Fizz1 (Peprotech, Rocky Hill, NJ) and anti-arg1 (Abcam, Cambridge, MA) at 1:100 dilution for M2-like markers. Primary antibodies used on THP-1 were: (1) monoclonal anti-CD11b (Abcam, Cambridge, MA) at 1:200 dilution for a pan-macrophage marker, (2,3) polyclonal anti-iNOS (Abcam, Cambridge, MA) at 1:100 and anti-TNFα (Abcam, Cambridge, MA) at 1:1000 for an M1-like marker, and (4,5) polyclonal anti-TGM2 (Abcam, Cambridge, MA) and anti-CD206 (Abcam, Cambridge, MA) at 1:1000 dilution for M2 markers. Cells were incubated in blocking solution

consisting of PBS, 0.1% Triton-X, 0.1% Tween-20, 4% goat serum, and 2% bovine serum albumin to prevent non-specific binding for 1 h at room temperature. Blocking solution was removed and cells were incubated in primary antibodies for 16 h at 4 °C. After washing in PBS, cells were incubated in fluorophore-conjugated secondary antibodies (Alexa Fluor donkey anti-rat 488 or donkey anti-rabbit 488, Invitrogen, Carlsbad, CA) for 1 h at room temperature. After washing again with PBS, nuclei were counterstained with 4'6-diamidino-2-phenylindole (DAPI) prior to imaging. Images of three 20× fields were taken for each well using a live-cell microscope. Light exposure times for ECM-activated macrophages were standardized based upon cytokine-activated macrophages (positive control). Exposure time was kept constant for each marker. CellProfiler (Broad Institute, Cambridge, MA, USA) was used to quantify images.

3.8 Western blot

Western blots were performed on treated macrophage cell lysates. Cell lysates were boiled at 95 °C for 5 min and electrophoresed on 4-20% gradient acrylamide gels. Specifically, 10 ug of protein was loaded into each well. Separated proteins were transferred to Polyvinylidene difluoride (PVDF) membranes (Bio-rad) using a wet transfer set up. Following transfer, membranes were then blocked for 45 min with Pierce protein-free blocking buffer (Pierce Chemical, Rockford, IL) and incubated overnight with the following primary antibodies: iNOS, TNF-α, STAT1, IRF3 CD206, TGM2, and KLF4, for THPI and iNOS, TNF-α, STAT1, Arg1, Fizz-1 and KLF4 for BMDM. Membranes were washed three times for 15 min in 1X PBS, before and after they were incubated with appropriate secondary antibody. The washed membranes were exposed to chemiluminescent substrate (Bio-Rad) and then visualized using chemidoc touch

instrument (Bio-Rad). Image Densitometry was evaluated using the shareware ImageJ (http://rsbweb.nih.gov/ij/index.html).

3.9 Nitric Oxide Quantification

Bone marrow macrophages and THP-1 cells were cultured and treated as previously described above. Following treatment, the supernatant from the wells was transferred to another plate and frozen at -80°C. The supernatant was thawed then 50 μL was added to another plate. 50 μL of standards consisting of sodium nitrite from 100 μM to 1.56 μM in a 1:2 serial dilution were added to the plate. The wells were treated with 50 μL of 1% sulfanilamide in 5% phosphoric acid for 10 minutes. Then 50 μL of 0.1% N-1-napthylethylenediamine [NED] dihydrochloride in water was added for an additional 10 minutes. The wells were then read at 540 nm and compared to the standard curve. Readings were normalized to the amount of DAPI-stained cells in each well as quantified by Cell Profiler.

3.10 Phagocytosis Assay

Following activation, cells were incubated with Vybrant Phagocytosis Kit FITC-labeled E. Coli beads for 2 hours. Wells were then washed once with 1XPBS and fixed with 2% paraformaldehyde for 30 minutes. Wells were washed 3 times with 1X PBS then stained with DAPI for 10 minutes and washed again three times with 1X PBS. Wells were imaged using an automated Live Cell Scope and quantified for mean intensity of the cells using Cell Profiler software. Mean intensity averages were calculated as percent changes from M0 naïve macrophage controls.

4. Discussion

The results of the present study show clearly that BMDM and THP-1 macrophages respond differently to the same stimulus. The phenotype of these two macrophage populations following activation by an ECM scaffold can appear very similar or vastly different depending upon whether gene expression, protein expression, or surface markers are evaluated. Furthermore, although SIS-ECM and UBM-ECM have both been associated with constructive, functional tissue remodeling outcomes in both pre-clinical animal models and in clinical applications in man, the macrophage phenotype resulting from activation with each ECM is distinct from those elicited by activation with either IFNγ+ LPS or IL-4. Finally, following activation by IFNγ + LPS, macrophages then exposed to degradation products of both ECM bioscaffolds show a marked downregulation of genes that are typically associated with an inflammatory profile.

ECM bioscaffolds typically promote a favorable tissue remodeling response when used to treat various types of soft tissue injury. This response includes the recruitment of endogenous stem cells, angiogenesis, and dampening of the inflammatory response. This pro-healing response has been attributed, in large part, to the effect of ECM on macrophage phenotype [8]. The objective of the present study was to comprehensively characterize the M_{ECM} phenotype. The source of macrophages used in this study; specifically, primary cells isolated from murine bone marrow (i.e., BMDM) and a transformed human mononuclear cell line (i.e., THP-1) are commonly used in studies to evaluate macrophage phenotype and behavior, and therefore the results are of interest to the field of macrophage biology [56, 57]. Since conclusions from such studies can have far-reaching implications, it is important to understand the effect of the source of macrophages upon study results.

4.1 BMDM and THP-1 macrophages differentially respond to the same stimulus

There was a clear difference between the response of BMDM and THP-1 macrophages to both canonical stimuli such as IFNy + LPS or IL-4 as well as to SIS-ECM and UBM-ECM degradation products. Gene expression analyses showed that THP-1 macrophages were not significantly affected by activation with IL-4, SIS-ECM, or UBM-ECM exposure, but there was a notable increase in most of the evaluated genes following activation by IFNγ+ LPS. This response was in stark contrast to the BMDM gene expression signature that showed significant changes to all of the applied stimuli. We hypothesize that these changes are likely due to the endogenous differences between a cell line (i.e., THP-1) and primary cells (i.e., BMDM). Another difference between the two macrophage populations was the difference in macrophage function following activation. THP-1 macrophages were associated with very little nitric oxide (NO) production and phagocytosis regardless of their stimulus, whereas BMDM showed an increase in NO production and phagocytic activity when stimulated with IFNy + LPS similar to the response one would expect in vivo following exposure to a pro-inflammatory stimulus.

The mechanism(s) by which macrophages are able to phagocytose and produce nitric oxide are largely unknown. However, the activation state of macrophages and the effect on cell function has been described [58, 59] and suggests that an increase in ARG1 and decrease in iNOS gene expression levels (as seen in Figure 1) may lead to the decrease seen in phagocytic activity [58]. Overall, the "M1-like" macrophages are more associated with phagocytosis and nitric oxide production than their "M2-like" counterparts. The present study shows that the challenged M_{ECM} phenotype has reduced phagocytic activity (associated with "M2-like") and an increase in nitric

oxide production (associated with "M1-like"). This finding is further evidence of the complexity

and plasticity of macrophages and the unique M_{ECM} phenotype compared to canonical controls.

The collective findings of the present study suggest that the in vitro response of BMDM respond

more similarly than THP1 cells with respect to the behavior observed by macrophages in

preclinical animal models and clinical studies that have examined ECM-mediated tissue

remodeling [10, 14]. Though there are multiple cell types utilized to investigate the in vitro

macrophage behavior, THP-1 macrophages were chosen because of their widespread use in the

immunology and regenerative medicine field [1-4]. The new data presented herein clearly show that

the THP-1 derived macrophages have a restrictive phagocytic and nitric oxide production

ability, thereby limiting "typical" macrophage functionality. There are also marked differences

in gene expression when compared to BMDM. Future work may benefit from using the results

reported herein as a comparative dada case for alternative cell types that may be used to

evaluate macrophage behavior in-vitro.

4.2 SIS-ECM and UBM-ECM induce similar but distinct macrophage phenotypes

Both SIS-ECM and UBM-ECM have been associated with an increased bioscaffold-localized

M2:M1 ratio in preclinical animal studies, and a constructive, functional tissue remodeling

response, but the macrophage phenotype has typically been characterized based upon a small

number of cell markers [62, 63]. In the present study, gene expression analysis of resting BMDM

showed that the macrophage response to SIS-ECM is similar to that of IL-4 activation. BMDM

exposed to UBM-ECM, in contrast, show a gene expression profile that is similar to that of the

$M_{IFN\gamma+LPS}$ phenotype. Several studies have investigated the mechanism by which macrophages are

activated in response to various stimuli, including the mechanism(s) of response to the canonical IFN + LPS stimulation ($M_{IFN\gamma+LPS}$) [64-72]. Following IFNγ+LPS stimulation, two pathways respond simultaneously. While LPS activates the TLR4 receptor, IFNγ activates IFNγR receptor [69, 73-77]. These different receptors activate separate pathways and therefore have different downstream targets. Through TLR4, LPS regulates NF-kB, AP-1, IRF-3 and IRF-5 which in turn affect HIF-1α, TNF, iNOS, IL-1, IL-12 and IL-6 [22, 78-80]. In contrast, IFNγ affects the JAK1/2, STAT1/2 pathways [22, 78-80]. These genes influence downstream iNOS, IL-12 and MHC II [22, 78-80]. Interestingly, the results of the present study show that in BMDM, UBM-ECM and SIS-ECM downregulate STAT1/2, IRF3/5 and iNOS. In addition, SIS-ECM facilitated downregulation of TNF-α. These results show the ability of ECM degradation products to regulate the activation mechanism of macrophages. The ability of ECM to activate macrophages is well established and not surprising; however the molecular composition of ECM is complex and the specific component(s) responsible for this effect are only partially understood. MBV, a bioactive component within the ECM only recently described, dramatically affect macrophage activation. We postulate that MBV may have a key role in the ECM-mediated effects upon macrophage activation. Furthermore, the differences between UBM-ECM and SIS-ECM may be due to different signature of cryptic peptides, matrix-bound vesicles (MBV) miRNA cargo or other components within the ECM[81].

However, when macrophages are first activated with a pro-inflammatory stimulus, both SIS-ECM and UBM-ECM down-regulate markers associated with a classic inflammatory response including iNOS, STAT1, STAT2, and KLF6 which is consistent with observed in vivo events. Both $M_{SIS-ECM}$ and $M_{UBM-ECM}$ augment nitric oxide production after IFNγ+LPS stimulus in vitro, but the $M_{UBM-ECM}$ phenotype is associated with an increased phagocytic capability compared to the $M_{SIS-ECM}$

phenotype. The present work shows that the "M$_{ECM}$" phenotype differs depending upon the ECM source tissue and is perhaps more accurately defined as "M$_{'source-tissue'-ECM}$", for example "M$_{SIS-ECM}$" or "M$_{UBM-ECM}$".

4.3 The effect of ECM upon naive macrophages vs. M$_{IFNy+LPS}$

Following injury, ECM scaffolds are applied to a soft tissue site following injury. The macrophages that interact with the ECM scaffold are likely in an activated state rather than a resting state. In an attempt to mimic this scenario, the present study evaluated the phenotypic response of both resting macrophages and IFNγ+LPS activated macrophages. There were clear differences between the response of "resting" macrophages and "pre-activated" macrophages to ECM degradation products. As previously mentioned, the M$_{SIS-ECM}$ and the M$_{UBM-ECM}$ phenotypes are distinct from each other when naive macrophages are exposed to degradation products of ECM. However, M$_{IFNy+LPS}$ activated macrophages respond similarly to both ECM sources with a down-regulation of inflammatory markers. The results of the present study show that the activation state of macrophages can influence the phenotypic response to subsequent stimuli. These findings are consistent with those of *in vivo* studies that show ECM bioscaffolds promote an anti-inflammatory macrophage phenotype with associated constructive and functional outcomes when utilized in response to injury or disease [8, 82]. The M$_{IFNy+LPS}$ may better represent a physiologic macrophage's state in response to injury, when investigating the in vitro response to a given stimulus.

5. Conclusions

The present study provides a comprehensive analysis of the macrophage phenotype associated with exposure to ECM scaffolds derived from the small intestine and urinary bladder. The results of the study demonstrate that the phenotype associated with both SIS-ECM and UBM-ECM is distinct from the canonical $M_{IFN\gamma+LPS}$ and M_{IL-4} phenotypes. Of note, there were also differences observed between SIS-ECM and UBM-ECM, suggesting that the microenvironment of the source tissue from which the ECM bioscaffold is produced also plays a significant role in determining patterns of macrophage activation. Lastly, it is noted that there are challenges and risks associated with making definitive conclusions about macrophage mediated events when results are based upon a particular macrophage population or a limited subset of macrophage markers. A greater understanding of the effect of macrophage phenotype upon the tissue remodeling process associated with ECM scaffolds will enhance both the design and associated production methods of such scaffolds materials, and would logically improve the clinical outcomes associated with their use.
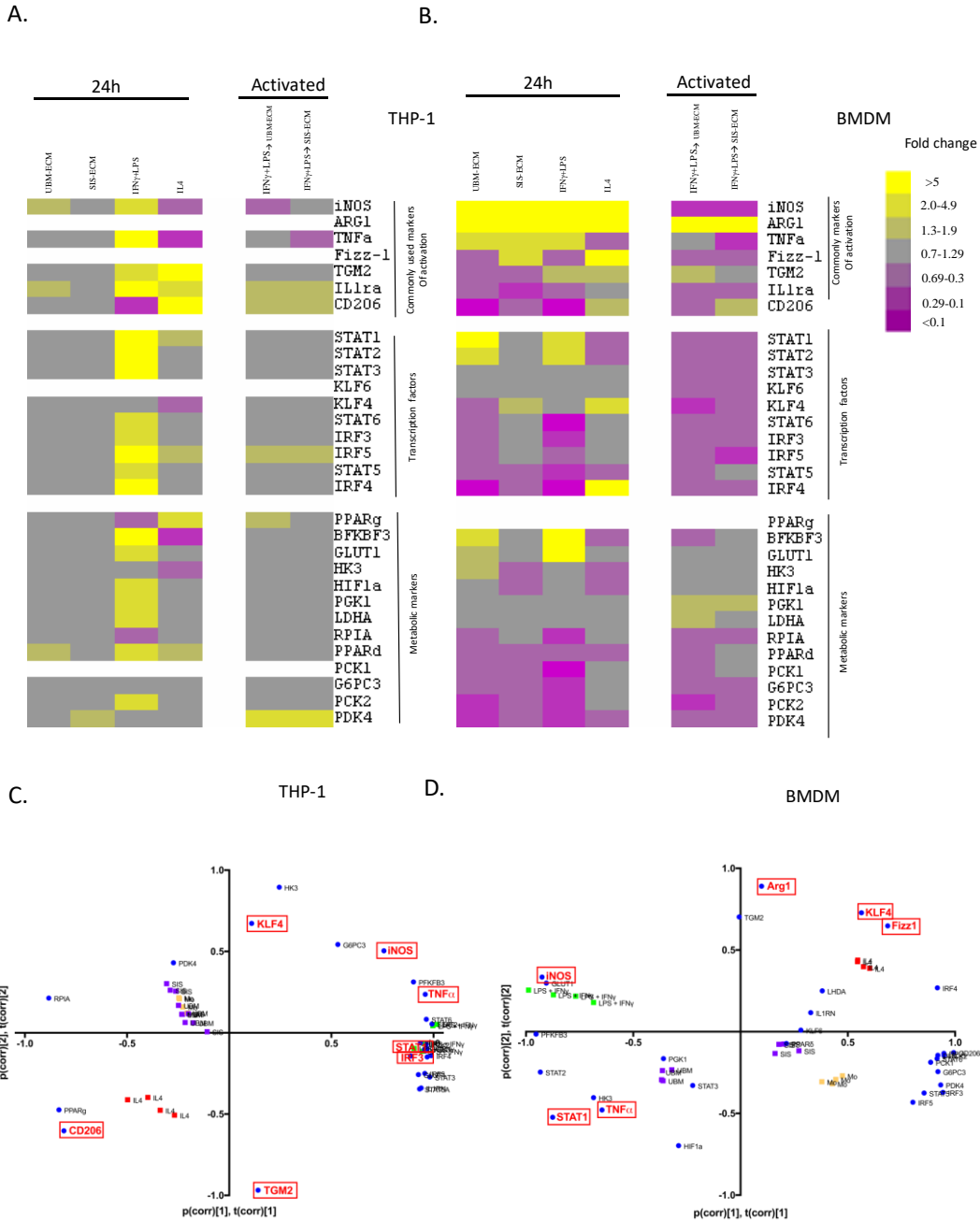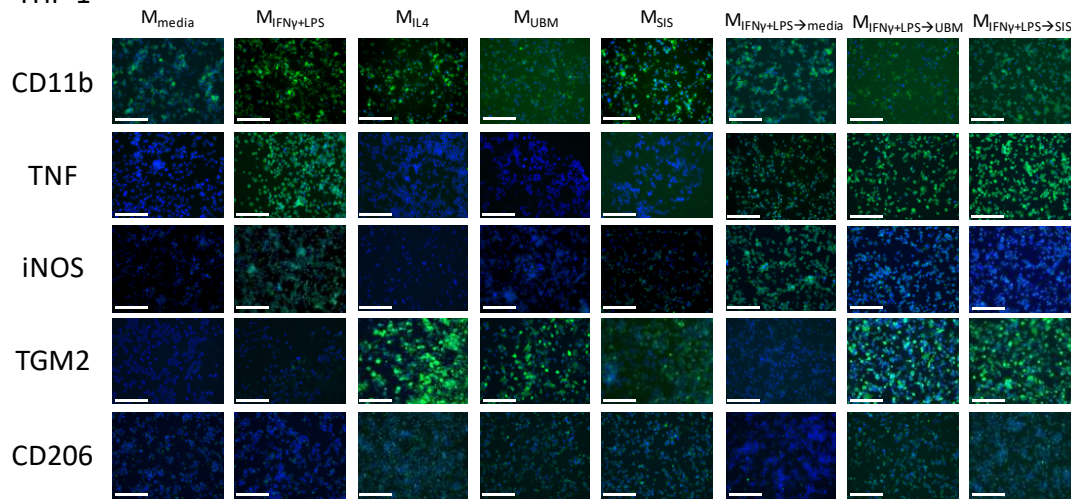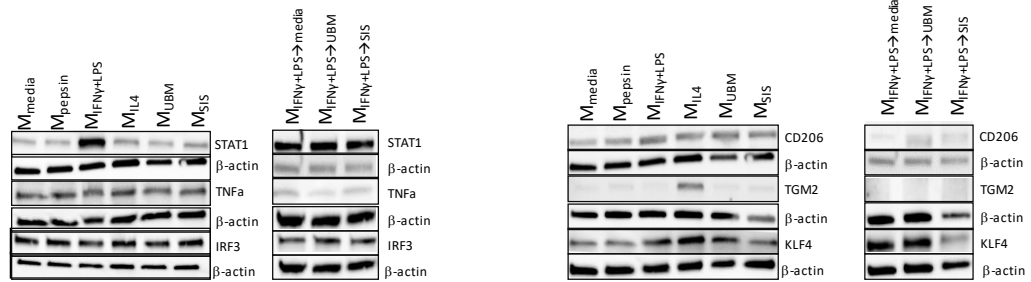
Figures

Figure 1. Gene expression of previously described "M1-like" and "M2-like" surface markers, cytokines, transcription factors and metabolic markers. (A) BMDM (left hand panel) and (B) THP-1 (right hand panel) were treated with, UBM-ECM, SIS-ECM, IFNγ+LPS and IL-4 for 24 hours. Additionally, macrophages were pre-treated with IFNγ+LPS for 6 hours followed by 24 hours of

UBM or SIS treatment (*n* = 3). Samples were normalized to media treatment. Gene expression was evaluated using qPCR data and is demonstrated in a heatmap form. Fold changes are presented using a color gradient bar. Principal component analysis (PCA) of delta Ct values scaled to unit variance. Biplot showing corrected principal component score values t(corr)[x] and loadings p(corr)[x] combined into one plot, where x is the component number. Genes that appear closer to the sample contributed to the distinction of that sample. The commonly cited genes associated with IFNγ+LPS and IL-4 activation that were chosen for further protein analyses for THP1 (C) and BMDM (D) derived macrophages and are highlighted.
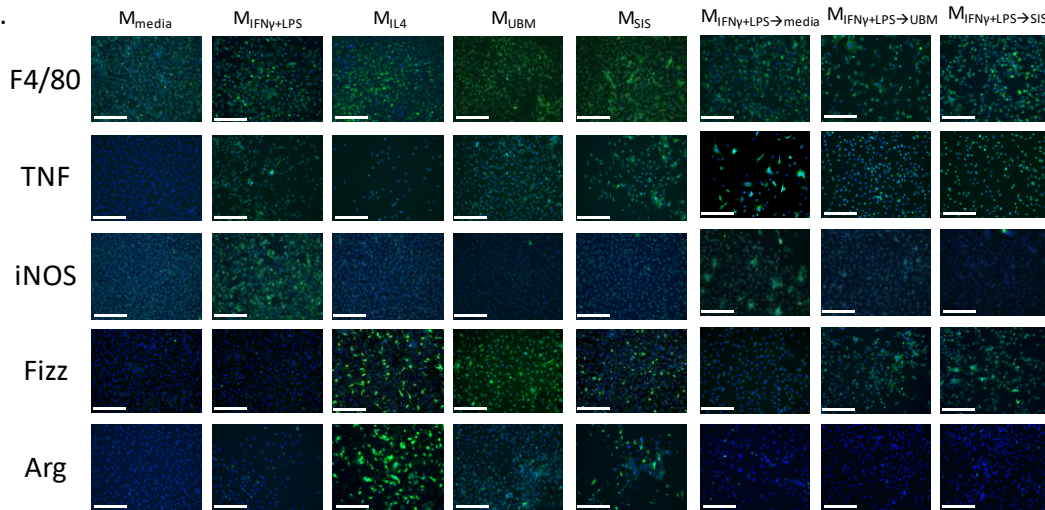
A.

THP-1

	M_media	M_IFNγ+LPS	M_IL4	M_UBM	M_SIS	M_IFNγ+LPS→media	M_IFNγ+LPS→UBM	M_IFNγ+LPS→SIS
CD11b								
TNF								
iNOS								
TGM2								
CD206								

B.

M_media | M_pepsin | M_IFNγ+LPS | M_IL4 | M_UBM | M_SIS

STAT1
β-actin
TNFa
β-actin
IRF3
β-actin

M_IFNγ+LPS→media | M_IFNγ+LPS→UBM | M_IFNγ+LPS→SIS

STAT1
β-actin
TNFa
β-actin
IRF3
β-actin

M_media | M_pepsin | M_IFNγ+LPS | M_IL4 | M_UBM | M_SIS

CD206
β-actin
TGM2
β-actin
KLF4
β-actin

M_IFNγ+LPS→media | M_IFNγ+LPS→UBM | M_IFNγ+LPS→SIS

CD206
β-actin
TGM2
β-actin
KLF4
β-actin

BMDM

C.

	M_media	M_IFNγ+LPS	M_IL4	M_UBM	M_SIS	M_IFNγ+LPS→media	M_IFNγ+LPS→UBM	M_IFNγ+LPS→SIS
F4/80								
TNF								
iNOS								
Fizz								
Arg								

D.

M_media | M_pepsin | M_IFNγ+LPS | M_IL4 | M_UBM | M_SIS

STAT1
β-actin
iNOS
β-actin
TNFa
β-actin

M_IFNγ+LPS→media | M_IFNγ+LPS→UBM | M_IFNγ+LPS→SIS

STAT1
β-actin
iNOS
β-actin
TNFa
β-actin

M_media | M_pepsin | M_IFNγ+LPS | M_IL4 | M_UBM | M_SIS

Arg
β-actin
Fizz
β-actin
KLF4
β-actin

M_IFNγ+LPS→media | M_IFNγ+LPS→UBM | M_IFNγ+LPS→SIS
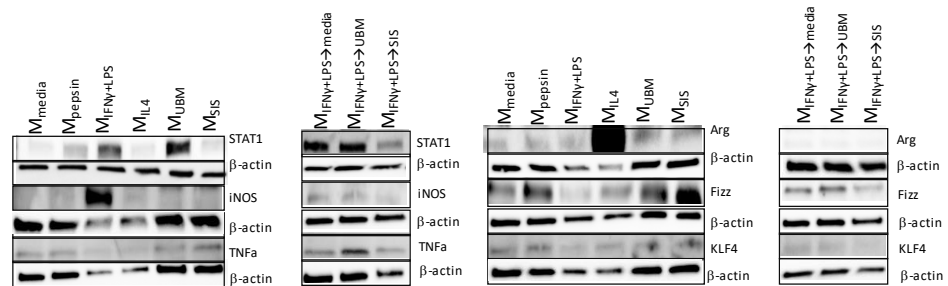
Arg
β-actin
Fizz
β-actin
KLF4
β-actin

Figure 2. ECM degradation products promote an immunomodulatory, "M2-like" phenotype. (A) Human monocytes from the THP1 cell-line were cultured in media supplemented with PMA to derive macrophages. Macrophages were treated with 20 ng/ml IFNγ and 100 ng/ml LPS to derive "M1-like" macrophages, 20 ng/ml IL-4 to derive "M2-like" macrophages, 200 ug/ml SIS-ECM degradation products, or 200 μg/ml pepsin control buffer. Additionally, "M1-like" macrophages were exposed to either 200 μg/ml UBM-ECM, or 200 μg/ml SIS-ECM degradation products to simulate the physiologic scenario of an injury treated with an ECM scaffold. Macrophages were fixed and immunolabeled for the pan-macrophage marker (CD11b), and strong indicators of the M1-like (TNFα and iNOS) and M2-like (CD206 and TGM2) phenotype. ECM treated cells show increased expression of TGM2 and CD206, markers associated with the IL-4-pushed phenotype. (B) Immunolabeling results were further evaluated using western-blot analysis of the TNFα, iNOS, CD206, and TGM2 markers (bottom Panel). (C) Bone marrow was isolated from C57bl/6 mice and cultured in media supplemented with macrophage-colony-stimulating-factor (MCSF) to derive macrophages. "M1-like" macrophages, "M2-like" macrophages, and ECM-activated macrophages were derived as described above. Additionally, "M1-like" macrophages were exposed to UBM-ECM or SIS-ECM degradation products as before. Macrophages were fixed and immunolabeled for the pan-macrophage marker (F4/80), and strong indicators of the M1-like (TNFα and iNOS) and M2-like (Fizz and Arginase) phenotype. ECM treated cells show increased expression of Fizz and Arginase, associated with the IL-4-pushed phenotype, as well as TNFα, associated with the IFNγ/LPS-pushed phenotype, suggesting that the ECM treated cells adopt a unique phenotype. (D).

Immunolabeling results were further evaluated using western-blot analysis of the STAT1, Arginase, Fizz, iNOS, and TNFα markers (bottom Panel). Scale bar is equal to 200 μm.
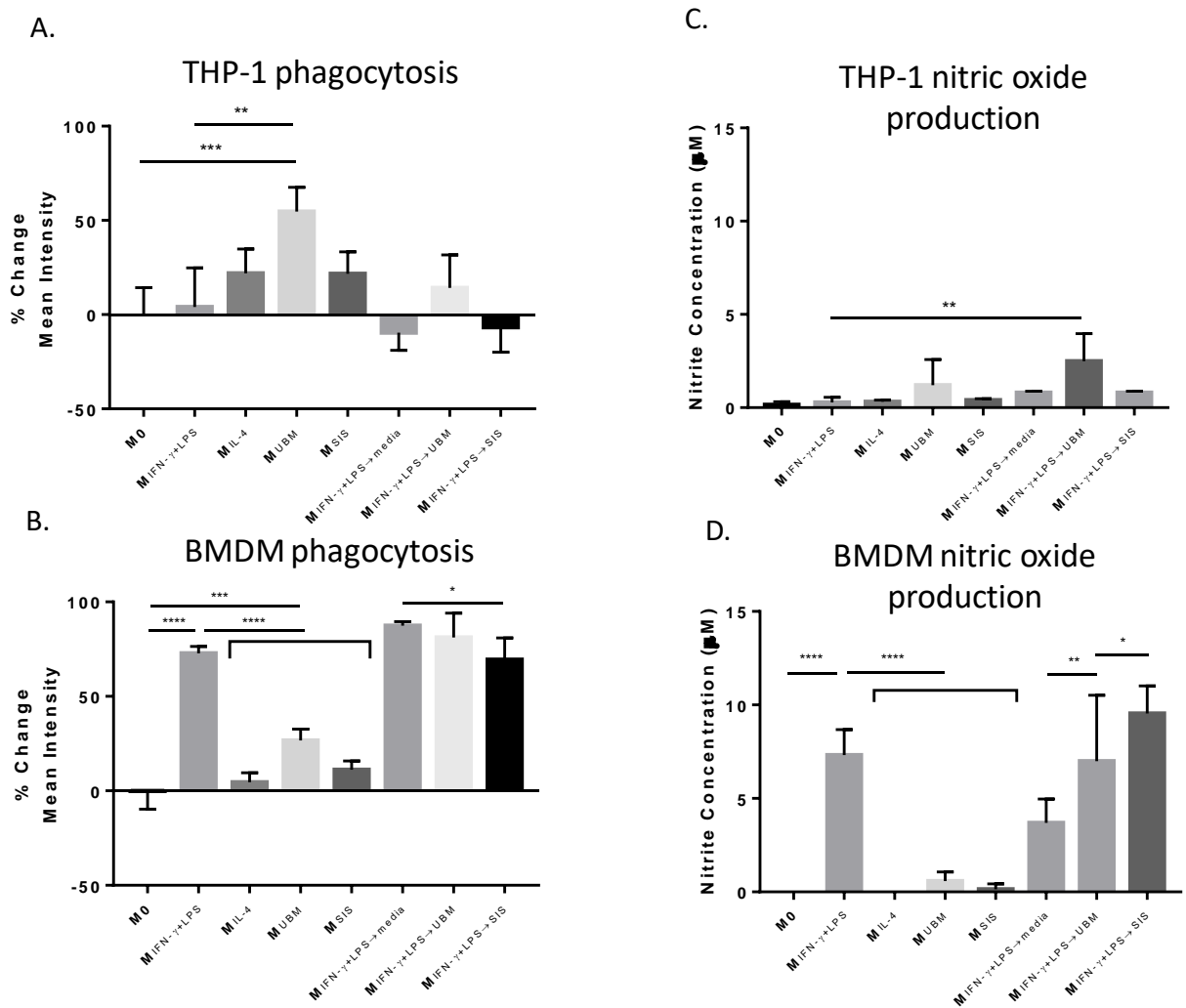
Figure 3. Functional Assessement of ECM-Treated Macrophages. Phagocytosis activity in BMDM

(A) and ThP1macrophages (B) was assessed using incubation with Vybrant FITC-labeled E. coli

particles then M.F.I. analysis. Nitric oxide production from BMDM (C) and THP1 macrophages (D)

was assessed using the Greiss reagent system on macrophage supernatants following treatment.

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