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Regulation of Macrophages by Extracellular Matrix Composition and Adhesion Geometry

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Abstract

The extracellular matrix is a dynamic structural component of tissue and plays a key role in wound healing by providing adhesive cues that regulate cell behavior during tissue repair. Macrophages are essential regulators of inflammation and tissue remodeling, and adaptively change their function in different microenvironments. Although much is known about how soluble factors including cytokines and chemokines influence immune cell function, much less is known about how insoluble cues including those presented by the matrix regulate their behavior. The goal of this study is to understand the potential role of different adhesive proteins and their geometric presentation in regulating macrophage behavior. Previously, using micropatterning and topographical features, we observed that macrophage elongation helps to promote polarization towards a pro-healing phenotype. In this work, we found that adhesion to different extracellular matrix ligands had only a moderate effect on macrophage cytokine secretion in response to prototypical activating stimuli. However, expression of arginase-1, a marker of pro-healing phenotype, was enhanced when cells were cultured on laminin, Matrigel, and vitronectin when compared to collagen, fibronectin, or fibrinogen. When micropatterned into lines, almost all matrix ligands allowed elongation of macrophages and a concomitant increase in arginase-1 expression. Together, these data demonstrate that extracellular matrix composition and adhesion geometry influence macrophage cell shape and function.

Lay Summary

In this study, we examined the effects of different extracellular matrix proteins on the phenotypic polarization of macrophages, a major innate immune cell involved in defense against pathogens, wound healing, and progression of many diseases. Our results suggest that the cytokine secretion response of macrophages to inflammatory or wound-healing stimuli was largely independent of the type of adhesion protein on which they were cultured, although laminin, Matrigel, and vitronectin promoted the expression of the pro-healing marker arginase-1. Interestingly, these matrix proteins are all prevalent in tumor environments, where pro-healing macrophages are often observed. Macrophages forced to elongate on patterned substrates of nearly all ECMs increased their expression of arginase-1. The extracellular matrix is dynamic in structure and composition during healing after injury and progression of many diseases including tissue fibrosis, cancer, or cardiovascular disease. This work may provide insight to how adhesion to different matrix environments and geometries regulates macrophages, and their impact on disease.

Keywords Extracellular matrix · Cell elongation · Macrophage · Polarization

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Introduction

Macrophages are essential regulators of pathogen defense, wound healing, and homeostasis, and adopt various phenotypes to perform these diverse functions in response to cues in their microenvironment [1]. At one end of the spectrum, macrophages adopt a pro-inflammatory phenotype in the presence of danger signals including interferon gamma (IFN-γ) and lipopolysaccharide (LPS), and secrete pro-inflammatory cytokines such as tumor necrosis factor (TNF-α) and express inducible nitric oxide synthase (iNOS) to promote immune defense. In contrast, macrophages in a wound healing microenvironment polarize towards a pro-healing phenotype, during which they secrete interleukin 10 (IL-10), and express arginase-1 to facilitate tissue repair [2, 3]. Although the effects of many different soluble cues including cytokines and chemokines on macrophage polarization have been well characterized [2–4], little is known about how insoluble cues including physical or adhesive properties of the environment regulate their behavior.

The extracellular matrix (ECM) serves not only as an architectural support for cells within tissues, but can also have an important signaling role through interactions with adhesion receptors and growth factors, among other molecules [5–10]. The ECM is dynamically remodeled during wound healing, as well as during diseases such as cancer, atherosclerosis, and fibrosis, among others. Upon injury, the wound healing process is initiated by the formation of the provisional matrix, composed of mainly fibrin, fibronectin (Fn), and vitronectin (Vn) [5, 11, 12]. At later stages, the matrix is remodeled and replaced by a collagen-rich matrix, which facilitates cell adhesion, contraction of tissues, and is also thought to regulate cellular function both directly and through interactions with growth factors [8, 13]. Changes in ECM architecture, including excess accumulation and crosslinking of collagen, are often considered one of the hallmarks of cancers [14–16] and fibrosis [17, 18]. Additionally, in vascular tissues, endothelial and smooth muscle cells reside within distinct ECM compositions, which are often altered during disease [19, 20]. Collagen type I (CI) has been shown to promote monocyte differentiation to macrophages and regulate macrophage matrix metalloproteinase (MMP)-9 production [21]. Despite dynamic changes in ECM during healing, how the composition and structure of the ECM regulates macrophage polarization and immune-mediated healing processes still remain largely unknown.

Earlier studies showed that ECM composition influences macrophage spread morphology upon differentiation from bone marrow cells [22]. ECM proteins including Fn, laminin (Ln), CI, and collagen type IV (CIV) were pre-coated on tissue culture plates before seeded with bone marrow cells. After differentiation, macrophages were well spread on CI and Fn-coated substrates, but remained rounded on CIV and Ln. Cells treated with IFN-γ and LPS assumed a circular, spread morphology and secreted similar levels of inflammatory cytokines regardless of the ECM coating. More recently, Zaveri et al. demonstrated adhesion to ECM proteins regulates macrophage phagocytosis, but not the inflammatory response to LPS [23]. Polystyrene micro-particles (MPs) coated with Fn were found to have enhanced uptake by macrophages when compared to Vn and fibrinogen (Fg) coatings. Consistent with the earlier study, levels of IL-6 and TNF-α secreted by macrophages were similar across all ECM coating conditions. Together, these studies found that while ECM composition may alter macrophage morphology and particle uptake, there was little effect on their inflammatory activation.

We recently demonstrated that cell shape modulates macrophage polarization. Specifically, cells that elongated along micropatterned lines of fibronectin enhanced their expression of markers associated with an anti-inflammatory phenotype [24]. In addition, macrophages cultured on grooved titanium or polymeric substrates aligned along grooves and increase their expression of anti-inflammatory markers both in vitro and after implantation in vivo [25]. Here, we wanted to explore how geometry of adhesion on different ECM ligands could influenced macrophage cell shape and function, particularly their polarization towards a pro-healing phenotype. To systematically test this, we first examined the effect of an array of ECM proteins on cell morphology and response to prototypical inflammatory (IFN-γ and LPS) and wound healing (IL-4 and IL-13) stimuli. We found that despite some changes in morphology on select ECMs, the cytokine secretion response to stimulation was largely similar across all matrix proteins tested. Interestingly, when we examined arginase-1 (Arg1) expression of cells cultured on different ECMs, we found significant differences, with higher levels observed on laminin, Matrigel, and vitronectin. Furthermore, patterning ECM into lines elicited an increase in the expression of Arg1 across almost all ECMs. Together, our results demonstrate that adhesion to different ECM ligands influences the expression of Arg1, and that elongation-induced changes in cell function are ligand independent.

Materials and Methods

Cell Isolation and Culture

All protocols involving animals were approved by the University of California Irvine's Institutional Animal Care and Use Committee, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal

Care International (AAALACi). Bone marrow-derived monocytes were isolated and cultured as previously described [26]. Briefly, bone marrow was isolated from femurs of 6–12-week-old C57BL/6 mice (Jackson Laboratories) and cells were collected, treated with ACK lysis buffer (Invitrogen), and resuspended and cultured in D10 media composed of high glucose DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/mL, 100 μg/mL streptomycin (all from Invitrogen), and 10% conditioned media containing macrophage colony-stimulating factor (M-CSF) produced by CMG 12-14 cells. After cultured for 7 days, bone marrow-derived macrophages (BMDM) were dissociated using Cell Dissociation Buffer (Invitrogen) and seeded on experimental substrates.

ECM Coating

All extracellular matrix (ECM) proteins were prepared following the manufacturer' s instructions. The concentrations of ECM proteins used for coating the wells were 100 μm/mL collagen type I (CI, rat; Corning) in 0.02 N acetic acid, 100 μm/mL collagen type IV (CIV, mouse; Corning) in 0.05 M HCl, 250 μm/mL fibrinogen (Fg, bovine; Sigma-Aldrich) in PBS, 20 μm/mL fibronectin (Fn, human; Corning) in H_2O, 20 μm/mL laminin (La, mouse; Corning) in high glucose DMEM, 150 μm/mL Matrigel GFR (Mg, mouse; Corning) in high glucose DMEM, and 10 μm/mL vitronectin (Vn, mouse; Abcam) in PBS. The ECM proteins were coated using a 1 mL solution per well of a 6-well plate overnight at 4 °C. Coated wells were washed thoroughly with their corresponding diluents prior to cell seeding. After 4 h of seeding, cells were stimulated with a combination of *E.coli* LPS (Sigma-Aldrich), recombinant murine IFN-γ (R&D systems), IL-4 (Invitrogen), and IL-13 (Invitrogen) with concentrations as described in the figure legends.

Cell Micropatterning

Micropatterned substrates were prepared as previously described [24]. Polydimethylsiloxane (PDMS; Dow Corning) stamps were replica-molded from silicon wafers with 20-μm microgrooves, which were fabricated using standard photolithography to make patterned stamps, or from petri dishes to make flat stamps. The stamps were sonicated for 10 min in 70% ethanol, washed thoroughly with ethanol, and then dried using N_2 air stream. The stamps were coated with ECM proteins at room temperature (RT) for 1 h and then washed thoroughly with their ECM corresponding diluent, and dried using a N_2 air stream. ECM proteins were transferred from the stamps to PDMS-coated substrates, which were treated with UV ozone (Jelight) for 8 min immediately prior to stamping. The stamped substrates were blocked with a 0.2% Pluronic F-127 solution (Sigma-Aldrich) at RT for 1 h, and washed with sterile PBS prior to cell seeding. After 2 h of cell seeding, substrates were washed thoroughly with DMEM to remove non-adherent cells and then transferred to new culture wells with fresh D10 media.

Cell Shape and Morphological Analysis

For analysis of cell morphology, cell images were acquired with a 20× objective on an Olympus inverted microscope, or EVOS microscope. The long axis was defined as the longest length of the cell while the short axis was determined as the length across the nucleus and perpendicular to the long axis. The long axis and the short axis of each cell were manually traced and measured using ImageJ software (National Institutes of Health). Inverted aspect ratio was calculated as the ratio of the short axis to the long axis, with ratios close to 1 corresponding to round cells and 0 indicating highly elongated cells.

Immunofluorescence Staining and Imaging

BMDMs were cultured on flat and patterned substrates as described above for 40 h before immunofluorescence staining. Cells were fixed with 100% cold methanol on ice for 15 min and then incubated with primary antibody, goat anti-arginase-I (Santa Cruz Biotechnology Inc.), for 1 h at RT. After cells were washed thoroughly with 1% BSA, they were incubated with secondary antibody, Alexa Fluor-594 donkey anti-goat (Jackson ImmunoResearch), for 1 h at RT. All cells were counterstained with Hoechst 3342 dye (Invitrogen). Fluorescent images were acquired using an Olympus inverted microscope with a 20× objective. Arg1 integrated intensity was quantified using CellProfiler software (MIT Broad Institute) using methods previously described [26].

Cytokine Secretion

BMDMs were cultured on ECM-coated substrates as described above for 24 h before cytokine secretion quantification. Supernatants were collected and analyzed for TNF-α and IL-10 by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (BioLegend, San Diego, CA).

Statistical Analysis

All data were presented as the mean ± SEM across at least three independent experiments, unless otherwise specified in the figure legends. Statistical analysis was performed using two-tailed Student's t test, or ANOVA, unpaired with Tukey's post hoc test as described in the figure legends. $p < 0.05$ was considered statistically significant.

Results

Characterization of Macrophage Morphology on Different ECM Ligands

To begin to examine the effect of the extracellular matrix (ECM) on macrophages, we cultured bone marrow-derived macrophages (BMDM) on a panel of ECM proteins including fibronectin (Fn), fibrinogen (Fg), collagen type I (CI), collagen type IV (CIV), laminin (Ln), Matrigel (Mg), and vitronectin (Vn). Cells were cultured on ECM-coated substrates with or without stimulation by LPS/IFN-γ, IL-4/IL-13, or IL-4/IL-13/LPS for 24 h. We found that BMDMs cultured on most ECM-coated substrates adhered and spread similarly within the same stimulation condition (Fig. 1a). Compared to the control, unstimulated condition, macrophages treated with IL-4/IL-13 elongated, but appeared spread and circular when treated with LPS/IFN-γ, similar to what we have observed previously [24]. BMDMs on Ln-coated substrates remained unspread and round regardless of stimulation condition (Fig. 1a). To more quantitatively assess cell shape, the degree of elongation was determined by measuring the width across the cell nucleus and dividing by the length of the longest axis, calculating an inverted aspect ratio. A ratio value close to 0 indicates a high degree of elongation, and a large ratio, close to 1, corresponds to a circular shape. We observed that across all but the LPS/IFN-γ stimulation condition, BMDM cultured on Ln substrates exhibited significantly higher inverted aspect ratio and reduced area when compared to other ECM substrates (Fig. 1, supplemental Tables 1 and 2). In addition, across all ECM conditions, except for Ln, macrophages stimulated with LPS/IFN-γ exhibited inverted aspect ratios in the range of 0.7 to 0.8, and cells stimulated with IL-4/IL-13 adopted elongated shapes with ratios between 0.25 and 0.37 (Fig. 1b, supplemental Table 1). Together, the data suggest that the effect of soluble factors on macrophage morphology was more potent than the ECM composition, which for the most part did not significantly influence changes in macrophage cell shape caused by soluble stimulation.

Effect of ECM Composition on Macrophage Cytokine Secretion

To examine the interactions between macrophages and ECM ligands on cell function, we characterized the secretion of TNF-α and IL-10, inflammatory and anti-inflammatory cytokines respectively in response to stimulation with combinations of LPS, IFN-γ, IL-4, and IL-13. Heatmap representation of the data showed that there were no significant differences in TNF-α concentration secreted by macrophages among the various ECM coating conditions (Fig. 2a). Moreover, secretion of IL-10 in response to IL-4/IL-13/LPS stimulation was similar also across different ECM coating conditions (Fig. 2a). To probe how TNF-α and IL-10 secretion might be correlated with cell shape, we plotted their average concentration against the average inverted aspect ratios for all matrix and stimulation conditions. Interestingly, the data suggest that TNF-α secretion was highly correlated to cell shape. More specifically, cells with high levels of TNF-α secretion, including those stimulated with LPS/IFN-γ (noted with blue symbols), exhibited inverted aspect ratios close to 1 (Fig. 2b). Macrophages stimulated with IL-4/IL-13 (shown with red symbols) secreted undetectable TNF-α levels and clustered close to 0 in inverted aspect ratios (Fig. 2b). Thus, elongated cells exhibit a reduced, less inflammatory response compared to round cells. We did not observe a correlation between IL-10 secretion and cell shape (Fig. 2b). Together, our data show that although cell shape and phenotype may be correlated in some cases, adhesion to different ECM ligands does not affect the secretion of TNF-α or IL-10 by macrophages in response to LPS, IFN-γ, IL-4, and IL-13, at least at the concentrations tested in this study.

Synergistic Effects of ECM Ligand and Adhesion Geometry on Macrophage Arginase-1 Expression

In our previous work, we found that macrophage elongation on fibronectin lines leads to enhanced Arg1 expression, a marker of pro-healing function in murine macrophages. To further examine the combined effect of ECM ligand and adhesion geometry on cell function, we micropatterned cells into 20-μm-wide line patterns using different ECM proteins, and cell shape and Arg1 expression were assessed by immunofluorescence. We observed that the cell shape of macrophages cultured on flat substrates was similar to what was found on ECM-coated polystyrene surfaces described above (Fig. 3a, c). Interestingly, on flat unpatterned surfaces, we found that the expression of Arg1 was varied in cells cultured on different ECMs. Specifically, Arg1 was highest in macrophages cultured on La, Mg, and Vn; moderate in cells cultured on CI and Fg; and lowest in cells on CIV and Fn (Fig. 3b). These trends were confirmed by Western blot (Fig. S1). With all ECMs, patterned substrates enhanced the degree of macrophage elongation when compared to their respective flat, unpatterned substrates (Fig. 3c). Furthermore, on almost all ECMs, elongation enhanced the expression of Arg1, with statistically significant increases on CIV and Fn (Fig. 3c). Macrophages cultured on Vn had the highest levels of Arg1 expression, and patterning-induced elongation did not further increase the expression levels. Together, these data demonstrate that adhesion to different ECM ligands has dramatic effects on the expression of Arg1, and adhesive geometries that elicit elongation enhance Arg1 expression across all ECMs.

Discussion

In this work, we screened the effects of different cell matrix proteins on macrophage function. We characterized

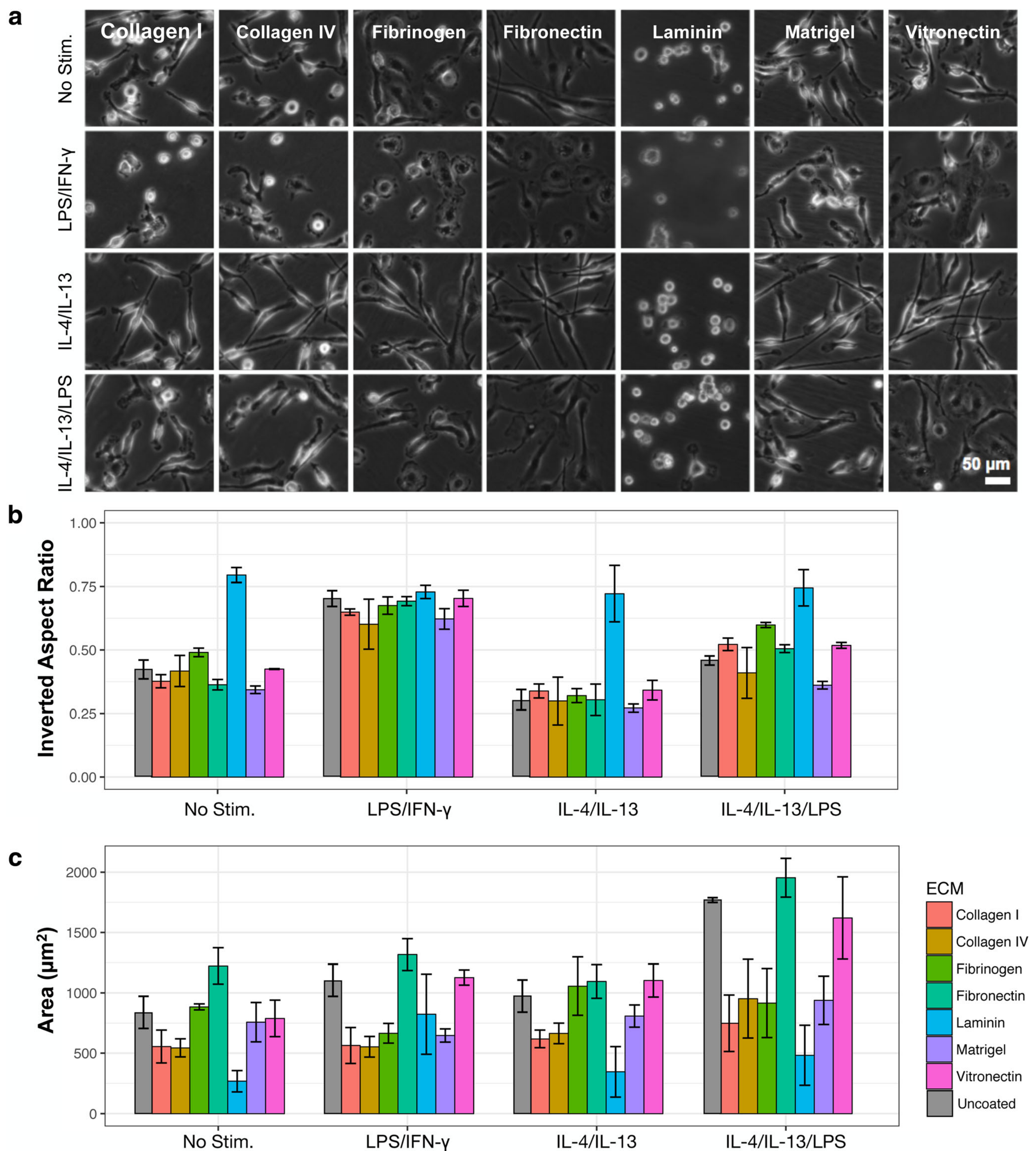

Fig. 1 Characterization of macrophage morphology upon activation on different ECM ligands. **a** Representative phase contrast images of BMDM on different ECM-coated or uncoated polystyrene substrates. Scale bar = 50 μm. **b** Quantified inverted aspect ratio defined as the ratio of the short axis to the long axis of cells. **c** Quantified area of BMDM cultured on different ECM-coated substrates. Mean ± SEM, $n = 3$ (except collagen IV, mean ± SD, $n = 2$). Cells were stimulated with indicated combinations of 1 ng/mL LPS, 10 ng/mL IFN-γ, 20 ng/mL IL-4, and 20 ng/mL IL-13

morphological changes in macrophages upon activation with soluble stimuli and also assessed functional changes via cytokine secretion and Arg1 expression. We observed that cell shape and cytokine secretion were more strongly modulated by the soluble factors, in comparison to the effects of ECM (Figs. 1 and 2), confirming what has been observed in other

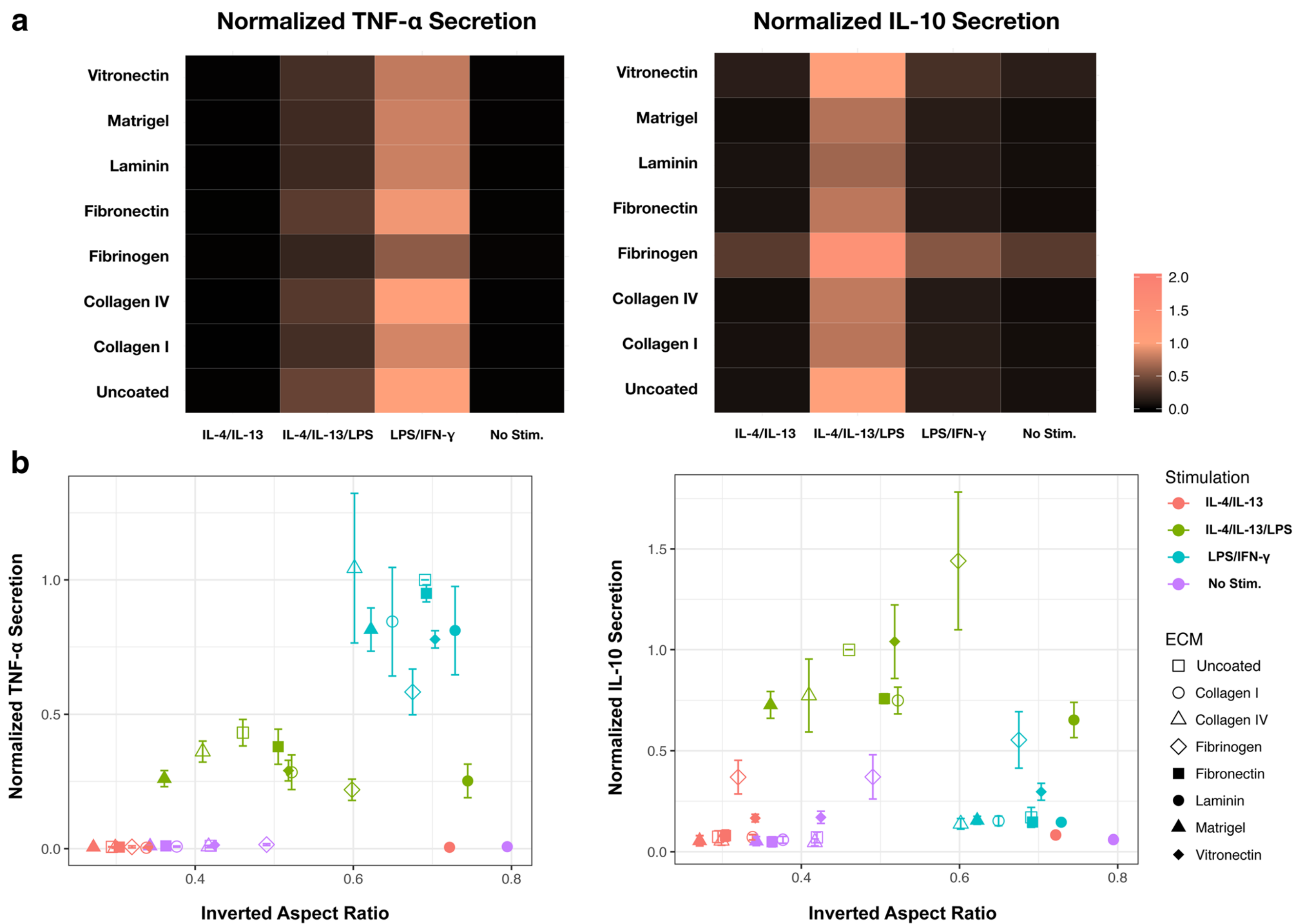

Fig. 2 Cytokine secretion from macrophages cultured on substrates coated with different ECMs. **a** Column-wise mean normalized heatmap of cytokines secreted by macrophages after culture on different ECM-coated or uncoated polystyrene substrates. TNF-α and IL-10 secretion by macrophages cultured on different substrates, normalized to the LPS/IFN-γ, and IL-4/IL-13/LPS-stimulated cells on uncoated substrates. **b** Graphs of TNF-α and IL-10 cytokine secretion versus macrophage inverted aspect ratio. Cells were stimulated with indicated combinations 1 ng/mL LPS, 10 ng/mL IFN-γ, 20 ng/mL IL-4, and 20 ng/mL IL-13

studies that had examined inflammatory activation [24, 27, 28]. However, the expression of a key alternative activation marker, Arg1, was strongly dependent on ECM composition, with Ln, Mg, Vn, and CI inducing higher expression compared to Fn, Fg, and CIV. Furthermore, adhesive geometries that increase cell elongation led to an increase in Arg1 expression across all ECM ligands, as we have previously observed using Fn [24]. This is consistent with work by our group and others examining the effects of topographical substrates on macrophage phenotype, where substrates that enhanced elongation led to a corresponding increase in markers associated to anti-inflammatory phenotypes [25, 26, 29]. Together, the data suggest that ECM composition and adhesion geometry both regulate macrophage behavior, and Arg1 expression in particular.

ECM remodeling and deposition of different matrix proteins during healing and disease may provide insight to the observed effects of ECM ligands on macrophage function. CI is found abundantly in tissues throughout the body, and intermediate levels of Arg1 were observed in macrophages cultured on this protein. Ln, Vn, and Mg have been associated with cancer, where macrophages typically adopt a pro-healing phenotype [30–32]. Since we observed that macrophages enhance their expression of Arg1 when cultured on these ECMs, our data suggest that the ECM itself may be playing a role in regulating the function of tumor-associated macrophages. CIV and Fn have been shown to increase in content with the progression of atherosclerotic lesions [33–35], in which macrophages exhibit mixed phenotypes [36, 37]. Nonetheless, low levels of Arg1 expression observed in macrophages cultured on these matrices may potentially be connected with high levels of macrophage inflammatory activation in cardiovascular disease. Finally, during wound healing, it is thought that the expression of Arg1 by macrophages facilitates a balance of collagen deposition and breakdown [13, 38, 39], and collagen composition is often altered in scars and keloids when compared to normal skin tissue [40]. Together, these

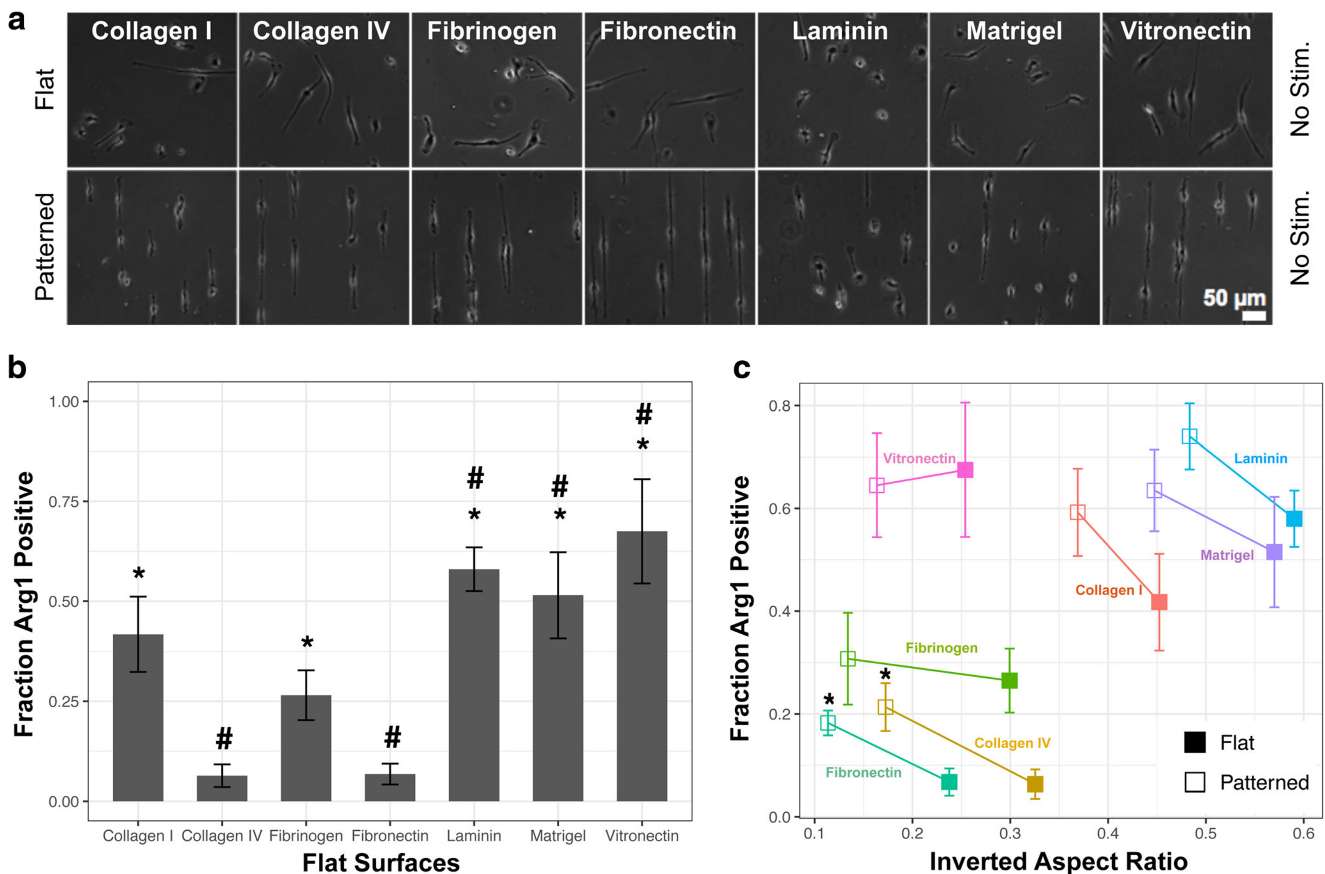

Fig. 3 The effect of ECM composition and adhesive geometry on BMDM arginase-1 (Arg1) expression. **a** Representative phase contrast images of cells cultured on different ECM substrates unpatterned or micropatterned with 20-μm-wide line. Scale bar = 50 μm. **b** Arg1 expression of macrophages cultured on different ECM-coated substrates. Mean ± SEM (*$p < 0.05$, compared to collagen IV or fibronectin-coated substrates; #$p < 0.05$, compared to fibrinogen-coated substrates, a paired two-tailed Student's t test, $n \geq 3$). **c** Graph of Arg1 expression versus macrophage inverted aspect ratio, cultured on different patterned and unpatterned ECM ligands. Mean ± SEM (*$p < 0.05$; an unpaired two-tailed Student's t test, patterned substrate compared to flat substrate for each ECM ligand, $n \geq 3$)

data suggest that a better understanding of the role of ECM protein composition, and their geometric presentation, on macrophage function is needed to fully comprehend the progression of many important pathological conditions.

The current study is limited to ECM ligands coated on a two dimensional surface, and further experiments will be needed to examine the effects of the matrix in more relevant three-dimensional (3D) culture models. 3D culture models may help reveal why CI, which forms a fibrillar architecture, and CIV, which forms part of the basement membrane, yield differential effects on Arg1 expression. Our previous work has shown that macrophages cultured on fibrin gels fabricated from combining Fg with thrombin inhibit their inflammatory activation, whereas Fg delivered in the soluble form led to inflammatory activation [41]. Here, we found only a moderate decrease in inflammatory activation and enhanced anti-inflammatory activity in cells cultured on adsorbed Fg, suggesting a potential role for ligand presentation—adsorbed, soluble, or polymerized—on its effects on macrophages. More detailed investigations of fibrillar ECM architectures, as well as mechanical cues associated with tissues are needed. Material stiffness and viscoelasticity are dramatically different in ECM hydrogels when compared to polystyrene or PDMS-coated surfaces, and these cues likely have an effect on macrophages, as has been reported [42–44]. Thus, future work will be focused on understanding how 3D matrix environments presented by physiological tissues influence macrophage inflammatory responses, and leveraging our knowledge to design better materials to encourage macrophage-mediated wound healing.

Conclusions

We demonstrate that ECM composition and adhesion geometry, together with soluble stimulation, play a role in regulating macrophage cell shape and function. When macrophages cultured on ECM ligands coated substrates including Fn, Fg, CI, CIV, Ln, Mg, and Vn were stimulated with soluble factors,

the effect of soluble factors on macrophage behavior was more potent than ECM ligand composition. When macrophages were cultured on ECM-coated PDMS substrates, cells adhered to Ln, Mg, and Vn ligands expressed higher levels of arginase-1 expression than Fn, CIV ligands. Finally, compared to flat substrates, ECM-patterned substrates enhance cell elongation level, and the expression of arginase-1 was increased when macrophages were cultured Fn, CIV-patterned substrates. Together, these results suggest the potential regulation of macrophage form and function by ECM ligands that influences tissue regeneration process. Further studies will be necessary to determine new material structures that encourage macrophage-mediated wound healing and reduced scar following tissue injury.

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Compliance with Ethical Standards

All protocols involving animals were approved by University of California Irvine's Institutional Animal Care and Use Committee, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALACi).

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