Modulation of macrophage phenotype by cell shape

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Phenotypic polarization of macrophages is regulated by a milieu of cues in the local tissue microenvironment. Although much is known about how soluble factors influence macrophage polarization, relatively little is known about how physical cues present in the extracellular environment might modulate proinflammatory (M1) vs. prohealing (M2) activation. Specifically, the role of cell shape has not been explored, even though it has been observed that macrophages adopt different geometries in vivo. We and others observed that macrophages polarized toward different phenotypes in vitro exhibit dramatic changes in cell shape: M2 cells exhibit an elongated shape compared with M1 cells. Using a micropatterning approach to control macrophage cell shape directly, we demonstrate here that elongation itself, without exogenous cytokines, leads to the expression of M2 phenotype markers and reduces the secretion of inflammatory cytokines. Moreover, elongation enhances the effects of M2-inducing cytokines IL-4 and IL-13 and protects cells from M1-inducing stimuli LPS and IFN-γ. In addition shape- but not cytokine-induced polarization is abrogated when actin and actin/myosin contractility are inhibited by pharmacological agents, suggesting a role for the cytoskeleton in the control of macrophage polarization by cell geometry. Our studies demonstrate that alterations in cell shape associated with changes in ECM architecture may provide integral cues to modulate macrophage phenotype polarization.

Macrophages play an essential role in innate immunity and are involved in a variety of immune functions, including host defense and wound healing. In addition, these cells participate in the progression of many chronic inflammatory diseases. To fulfill their functionally distinct roles, macrophages are capable of polarizing toward a spectrum of phenotypes, which include classical (proinflammatory, M1) and alternative (anti-inflammatory, prohealing, M2) activation states, as well as a regulatory phenotype and subtypes of these broad classifications (1). In the presence of inflammatory stimuli and danger signals, macrophages polarize toward the M1 state and release reactive species and inflammatory cytokines to fight pathogens. In contrast, a wound healing environment promotes polarization toward an M2 phenotype and leads to cellular processes that facilitate tissue repair. Although distinct macrophage subpopulations are clearly observed at different phases of the immune response to infection and injury (2), regulation of phenotypic polarization of these remarkably plastic cells still remains poorly defined.

Activated macrophages are derived from circulating monocytes or resident tissue macrophages and migrate through the extracellular space in response to chemotactic agents to reach the target wound or infection site. Although it is thought that cytokines and chemokines are the primary regulators of macrophage behavior (3), some recent studies suggest that tissue structure and physical cues in the extracellular environment also contribute to their function (4). In the context of tissue healing, deposition of the provisional ECM and collagen remodeling may indeed influence macrophage polarization. In support of this, different macrophage subpopulations in vivo have been shown to exist within specific tissue architectures. For example, during atherosclerosis, although both M1 and M2 phenotypes are present, M2 cells dominate within the collagen-rich fibrous cap and adventitia surrounding the plaque and exhibit an elongated morphology (5, 6). Furthermore, it was recently shown using intravital imaging that macrophages align and migrate along collagen fibrils within a tumor (7). Despite evidence suggesting that macrophages adopt different geometries in vivo, how these changes in cell shape might feed back to regulate their functional phenotype has not been previously explored.

Cell shape changes have been associated with different functional states of cells (8), including proliferation and apoptosis (9), nuclear organization (10), stem cell differentiation (11, 12), and muscle cell contractility (13). It is thought that interactions between the ECM, cell surface adhesion proteins, and cytoskeleton, as well as subsequent changes in intracellular contractility, are important in mediating shape-induced effects (14). Although these molecular structures are known to be key mediators of mechanotransduction in many cell types, their roles in sensing physical cues in the macrophage microenvironment and regulating macrophage polarization state are not known.

Here, we investigate the role of cell elongation, which is observed in cells within fibrous tissue architectures, on the polarization of macrophages. We observed that macrophages exhibit different degrees of elongation when stimulated toward M1 or M2 phenotypes with cytokines in vitro. Using a micropatterning approach to control the shape of cells directly, we found that elongation of cells induced polarization toward an M2 phenotype. Moreover, elongation enhanced the effect of M2-inducing cytokines and inhibited the effect of M1-inducing cytokines, suggesting that cell shape plays an important role in modulation of the phenotypic polarization of macrophages. Transduction of cell shape to changes in macrophage phenotype was dependent on contractility within the actin cytoskeleton, because pharmacological inhibition of actin or myosin prevented polarization by cell shape. Shape-induced cell polarization may play a critical role in modulation of macrophage phenotype by means of changes in ECM structure during wound healing and tissue repair.

Significance

Macrophages are central regulators of the immune response during infection and wound healing, and their behavior is largely thought to be regulated by soluble factors in the microenvironment. Here, we find that adhesive cues are also critical for regulating the proinflammatory vs. prohealing state of these cells. We use engineered cell culture substrates to control cell shape and find that elongation of cells promotes a prohealing phenotype. Moreover, we identify key molecular pathways that are responsible for transducing physical cues to the biochemical cues that govern this response. Together, these studies suggest an important role for cell shape in regulating macrophage function.

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Results

Macrophage Polarization State Is Associated with Changes in Cell Shape. We observed that bone marrow-derived macrophages (BMDMs) stimulated in culture with cytokines to induce M1 or M2 polarization displayed markedly different cell morphologies, as has also been reported by other groups (15, 16). Addition of LPS and IFN-γ, which stimulate M1 polarization, caused cells to flatten into a round, pancake-like shape within 24 h of stimulation. In contrast, addition of IL-4 and IL-13, which stimulate M2 polarization, led to cellular elongation (Fig. 1*A*). Quantification of the degree of cell elongation, which we defined as the length of the longest axis divided by the length of the short axis across the cell nucleus, showed that M2 cells exhibited a significantly higher degree of elongation compared with either M1 cells or unstimulated M0 cells (Fig. 1*C*). However, the spread cell area remained constant across all three macrophage populations (Fig. 1*B*). To confirm the macrophage polarization state, we performed immunofluorescence staining and Western blotting to evaluate the expression of inducible nitric oxide synthase (iNOS) and arginase-1, which are established markers of proinflammatory and prohealing phenotypes, respectively. We found that macrophages stimulated with LPS/IFN-γ expressed high levels of iNOS but not arginase-1, whereas cells stimulated with IL-4/IL-13 expressed high levels of arginase-1 but not iNOS (Fig. 1 *D* and *E*). Arginase-1 expression was dependent on the dosage of IL-4/IL-13, and the extent of cell elongation correlated with the level of arginase-1 expression (Fig. 1 *F* and *G*). Together, these data suggest that cell shape is associated with the macrophage polarization state and that M2 polarization correlates with an increased degree of cell elongation.

Macrophage Elongation Stimulates Polarization Toward an M2 Phenotype. To explore whether cell shape may play a direct role in the phenotypic polarization of macrophages, we used a micropatterning approach to control the geometry of cell adhesion (Fig. 2*A*). Macrophages were cultured on micropatterned substrates containing 50- or 20-μm wide lines of fibronectin separated by 20-μm wide lines coated with Pluronics F127 (Sigma), a nonadhesive surface that prevents cell adhesion (17). Cells adhered and spread along the fibronectin lines and did not migrate or spread to the regions coated with Pluronics F127 (Fig. 2*B*). We found that the degree of cell elongation was highest when cultured on 20-μm wide lines, where they elongated to a similar degree as IL-4/IL-13–stimulated cells, whereas cells cultured on 50-μm wide lines were not more elongated than cells on control, unpatterned surfaces (Fig. 2*D*). The degree of cell spreading remained constant in all patterned and unpatterned conditions (Fig. 2*C*). We next examined whether changes in cell elongation, as controlled by micropatterning, induced changes in expression of macrophage phenotype markers. Cells were cultured on unpatterned and patterned (50-μm and 20-μm wide lines) surfaces for 24 h, and iNOS expression and arginase-1 expression were evaluated by Western blotting. Interestingly, we found that cells cultured on 20-μm wide lines expressed significantly higher levels of arginase-1 compared with cells cultured on 50-μm wide lines or unpatterned cells, whereas all three conditions exhibited similarly low levels of iNOS expression (Fig. 2*E*). These data suggested that cell elongation promotes macrophage polarization toward a prohealing, M2 phenotype and does not influence inflammatory activation.

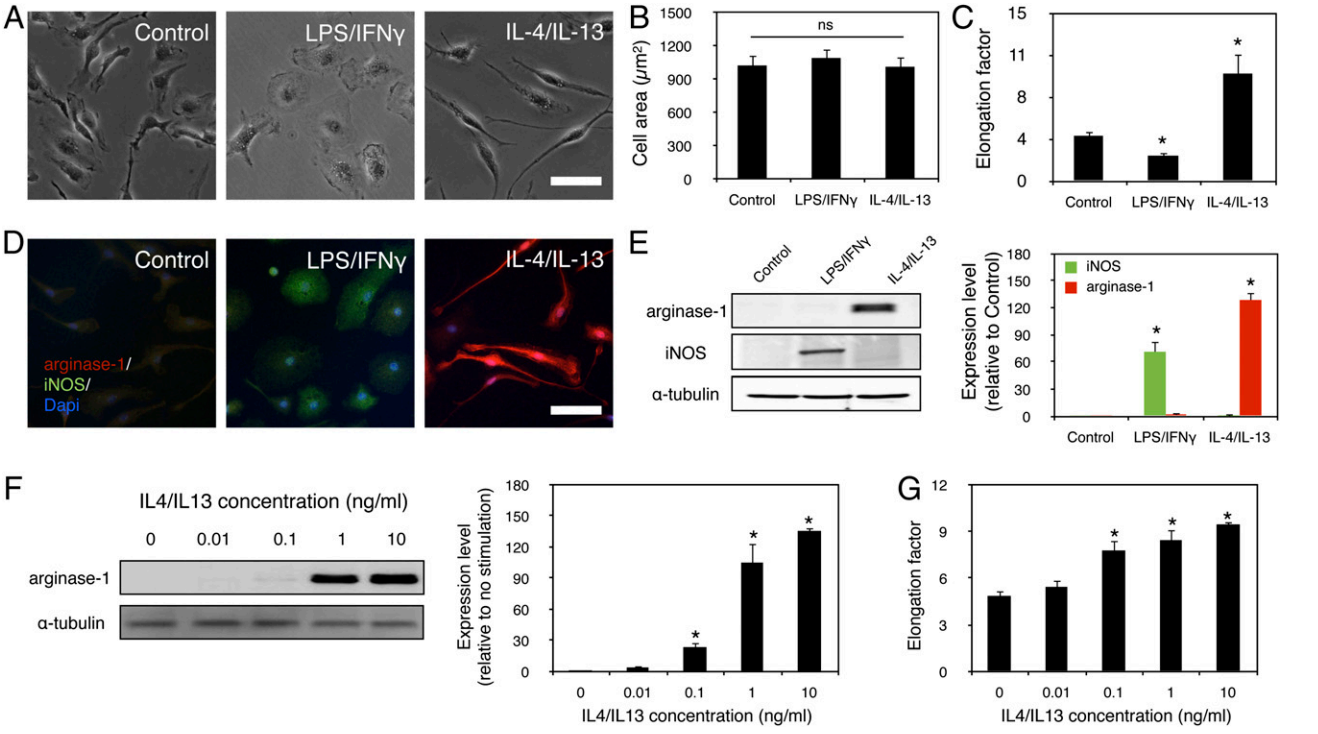

Fig. 1. Polarization of macrophages toward an M2 phenotype is associated with an elongated cell shape. (*A*) Phase contrast images of BMDMs left untreated (*Left*) or treated with LPS/IFN-γ (*Center*) or IL-4/IL-13 (*Right*). (Scale bar: 50 μm.) (*B*) Quantification of elongation, or length of the long axis divided by length of the short axis, for control, LPS/IFN-γ–treated, and IL-4/IL-13–treated cells. (*C*) Quantification of area for control, LPS/IFN-γ–treated, and IL-4/IL-13–treated cells. (*D*) Fluorescence images of cells immunostained for iNOS (green) and arginase-1 (red) and Hoechst nuclear counterstain (blue) of control, LPS/IFN-γ–treated, and IL-4/IL-13–treated cells. (Scale bar: 50 μm.) (*E*) Representative Western blot of iNOS, arginase-1, and α-tubulin of control, LPS/IFN-γ–treated, and IL-4/IL-13–treated cells and quantification of average across three separate experiments. (*F*) Representative Western blot of arginase-1 and α-tubulin in response to different dosages of IL-4/IL-13 and quantification of average across three separate experiments. (*G*) Quantification of cell elongation for macrophages treated with different IL-4/IL-13 dosages. Error bars indicate the SEM for three separate experiments. $^*P < 0.05$ compared with control cells as determined by the Student *t* test. ns, not significantly different.

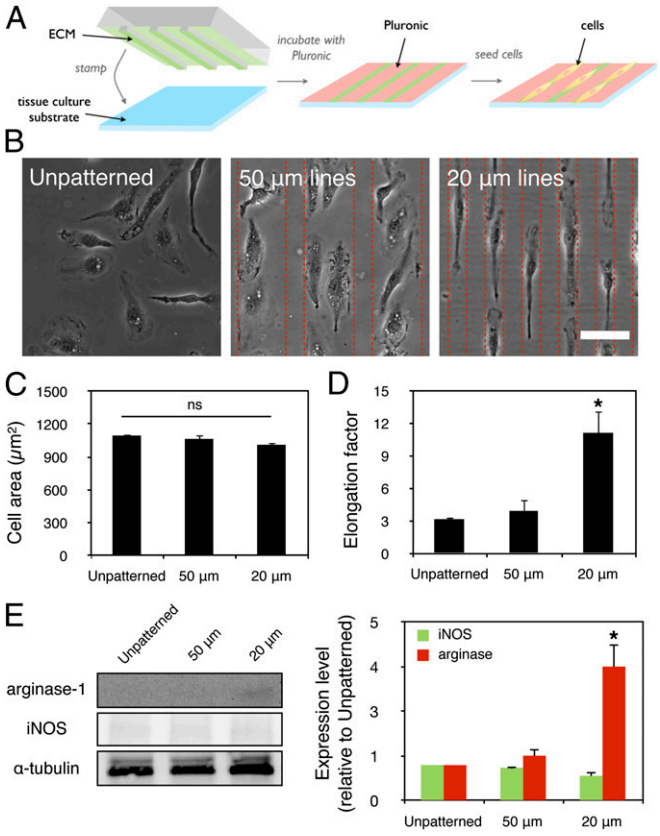

Fig. 2. Elongation of cells by micropatterning drives macrophage polarization. (*A*) Schematic of method used to micropattern cells by microcontact printing of arrays of fibronectin lines of various widths. (*B*) Phase contrast images of unpatterned cells and cells patterned on 50-μm and 20-μm wide lines. (Scale bar: 50 μm.) (*C*) Quantification of cell area of unpatterned cells and cells patterned on 50-μm and 20-μm wide lines. (*D*) Quantification of elongation factor of unpatterned cells and cells patterned on 50-μm and 20-μm wide lines. (*E*) Representative Western blot of iNOS, arginase-1, and α-tubulin of unpatterned and patterned cells and quantification across three separate experiments. Error bars indicate the SEM for three separate experiments.*$P < 0.05$ compared with unpatterned cells as determined by the Student *t* test. ns, not significantly different.

To examine further how cell elongation influences macrophage phenotype and function, we evaluated arginase-1, as well as CD206 (macrophage mannose receptor) and YM-1 by flow cytometry. Cells were seeded on unpatterned and patterned (20-μm wide lines) surfaces for 24 h; they were then removed from the substrate and stained with primary and fluorescently labeled secondary antibodies. We found a significant up-regulation of arginase-1, CD206, and YM-1 macrophage markers in cells cultured on patterned surfaces compared with cells on unpatterned surfaces, although the expression of all three markers was not as high as levels achieved with IL-4/IL-13 cytokine stimulation (Fig. 3 *A*–*C*). Furthermore, upon analysis of the cytokines secreted from patterned and unpatterned cells, we found that elongation of cells in general reduced the secretion of proinflammatory cytokines, including CD54, IFN-γ, and macrophage inflammatory protein-1α (Fig. 3*D*).

Together, these results demonstrate that cell elongation indeed stimulates polarization toward an M2 phenotype, enhancing markers associated with a prohealing phenotype and diminishing secretion of proinflammatory cytokines. These data suggest that cell shape, independent of soluble cues, plays a role in the regulation of the phenotypic polarization of macrophages.

Macrophage Elongation Synergizes with Cytokines to Regulate Polarization. Given that macrophages are also likely to be subjected to cytokine stimulation in their native in vivo microenvironment, we wanted to examine the combined effect of cell shape and soluble stimuli. Macrophages were first seeded onto unpatterned and micropatterned (20-μm wide lines) surfaces overnight, and they were then incubated for an additional 24 h with low doses of IL-4/IL-13, which were found to cause undetectable or low levels of arginase-1 expression (Fig. 1*F*). We found that elongation of cells indeed enhanced the effects of IL-4/IL-13 stimulation. For each dosage of IL-4 and IL-13, the expression of arginase-1 was higher in patterned cells compared with unpatterned cells (Fig. 4*A*), suggesting that elongation synergizes with IL-4/IL-13 to enhance M2 polarization. However, when patterned and unpatterned cells were treated with IFN-γ/LPS to stimulate iNOS expression, we found that patterned cells that were stimulated with

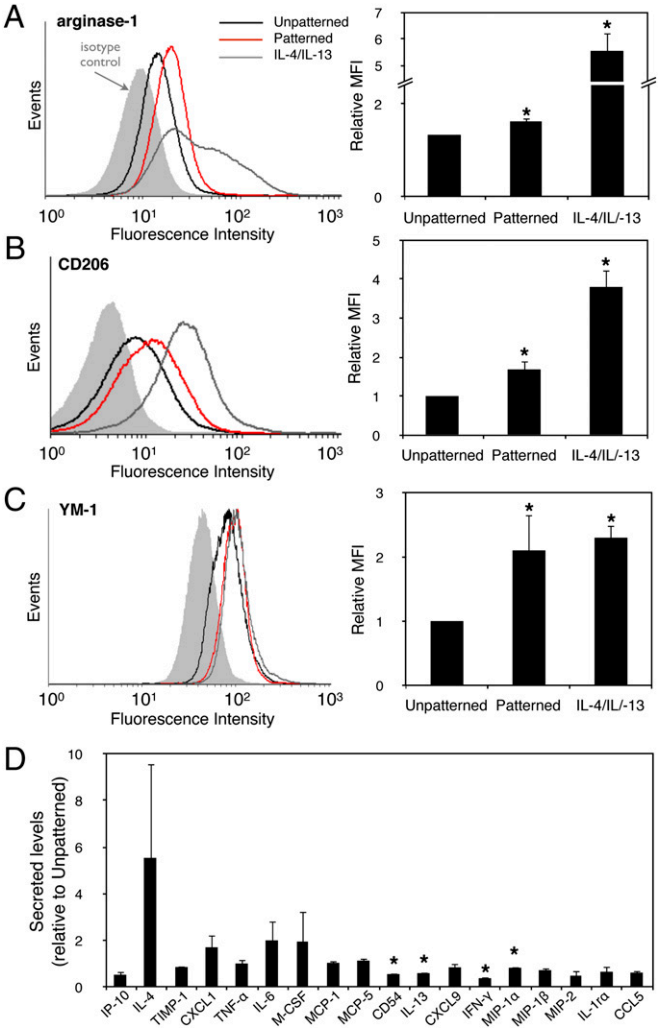

Fig. 3. Macrophage elongation up-regulates markers of M2 polarization and reduces secretion of proinflammatory cytokines. Representative flow cytometry histograms (*Left*) and averaged relative mean fluorescence intensity (MFI) across three separate experiments (*Right*) of arginase-1 (*A*), CD206 (*B*), and YM-1 (*C*) for unpatterned cells and cells patterned on 20-μm wide lines and for cells treated with IL-4/IL-13. (*D*) Graph of secreted levels of indicated cytokines for patterned cells relative to unpatterned cells. Error bars indicate the SEM for three separate experiments. *$P < 0.05$ compared with unpatterned cells as determined by the Student *t* test. CCL5, chemokine (C-C motif) ligand 5; CXCL, chemokine (C-X-C motif) ligand IP-10, interferon gamma-induced protein 10; MCP, monocyte chemotactic protein; M-CSF, macrophage-CSF; TIMP-1, TIMP metallopeptidase inhibitor 1.

1 ng/mL IFN-γ/LPS exhibited significantly lower levels of iNOS compared with unpatterned cells (Fig. 4*B*). These data demonstrate that cellular elongation protects macrophages from M1 polarization by inflammatory stimuli. Finally, we examined whether restricting macrophages from spreading by patterning on 50- × 50-μm discrete square adhesive islands prevents the up-regulation of arginase-1 by IL-4/IL-13 stimulation. We found that cells that were restricted from spreading exhibited lower levels of arginase-1 compared with cells cultured on unpatterned surfaces and administered the same amount of IL-4/IL-13 (Fig. S1), suggesting that IL-4/IL-13–mediated polarization requires, at least in part, the elongation of cells.

Together, our data show that elongation enhances the expression of arginase-1 induced by IL-4/IL-13 and mitigates the expression of iNOS induced by IFN-γ/LPS. Moreover, preventing cell elongation inhibits the full activation of the M2 polarization program induced by IL-4/IL-13.

Shape-Induced Polarization of Macrophages Requires Cytoskeletal Contractility. To begin to explore potential molecules that might transduce cell shape into biochemical signals that regulate macrophage polarization, we examined the actin cytoskeleton, which has been implicated in transducing shape in other cell types (11, 13, 18). Unpatterned, patterned, and cytokine-stimulated macrophages were fixed and stained with phalloidin, and then evaluated by immunofluorescence microscopy. We found that cells stimulated with IL-4/IL-13, as well as patterned cells, exhibited higher fluorescence signal compared with control unpatterned, unstimulated cells or LPS/IFN-γ–stimulated cells (Fig. 5*A*). The unpatterned, unstimulated cells exhibited clusters of actin or podosomes, which were not observed in the other conditions. We further examined the effect of pharmacological inhibitors that prevent actin and actin-associated signaling pathways. Cells were incubated with blebbistatin to inhibit myosin phosphorylation, cytochalasin D to inhibit actin polymerization, Y27632 to inhibit Rho-associated kinase (ROCK), and ML-9 to inhibit myosin light chain kinase (MLCK), and actin cytoskeleton was evaluated by phalloidin staining. We found that the level of phalloidin intensity was generally reduced with treatment of inhibitors in unpatterned cells, patterned cells, and cells treated with IL-4/IL-13 (Fig. 5*B*).

We then asked whether actin and actin-associated contractility is required for shape-induced macrophage polarization. We developed an immunofluorescence cytometry method to evaluate arginase-1 expression by microscopy and confirmed that the results from immunofluorescence cytometry correlated well with the data obtained by flow cytometry (Fig. S2). Cells were seeded on unpatterned and patterned surfaces, treated with the aforementioned drugs to inhibit actin and actin-associated contractility, and then evaluated for cell shape and arginase-1 expression. The degree of spreading was not affected and cells were still able to achieve significant elongation on patterned surfaces when cultured in the presence of drugs (Fig. 5 *C* and *D*). However, treatment with all drugs abrogated the up-regulation of arginase-1 in patterned cells and led to expression levels similar to those of unpatterned cells (Fig. 5*E*). For the most part, inhibition of cytoskeletal contractility did not affect IL-4/IL-13–induced changes in cell shape or arginase-1 expression, suggesting that although IL-4/IL-13 appears to alter the cytoskeleton, cytoskeletal contractility is not required for cytokine-induced macrophage polarization. These data suggest that actin polymerization and myosin-dependent cytoskeletal contractility are required for macrophages to sense shape and mediate shape-induced polarization, and that cytokine-induced and shape-induced polarization pathways are likely to be distinct.

Discussion

In this study, we revealed a role for cell elongation in the regulation of macrophage phenotype polarization. We found that the elongation of macrophages led to higher levels of arginase-1, CD206, and YM-1, which are markers of M2 polarization. Although elongation had no effect on the expression of iNOS, a marker of M1 polarization, the secretion of proinflammatory cytokines was reduced. The degree of elongation required to induce expression of M2 markers was similar to the elongation levels elicited by IL-4/Il-13 stimulation. M2 marker expression stimulated by cell shape was not as high as expression in cells stimulated with high doses of cytokines, but patterned cells stimulated with a low dose of IL-4/IL-13 exhibited higher levels of arginase-1 expression compared with unpatterned cells subjected to the same soluble conditions. Together, these results suggest that cell shape itself polarizes macrophages toward an M2 phenotype and that cell shape synergizes with cytokines to enhance their effects on polarization state.

Macrophages are instrumental in facilitating collagen deposition during wound healing (19), and it is thought that arginase-1, an enzyme that metabolizes arginine to form proline, is involved in this process. Interestingly, however, it has also been shown that expression of arginase-1 prevents excessive collagen deposition by depleting arginine, which is needed for T cell-mediated inflammation and fibrosis (20). Together, these data suggest that tight control of arginase-1 levels is likely needed to achieve an optimal wound healing response with minimal fibrosis or scar formation. Our study suggests that changes in the physical extracellular

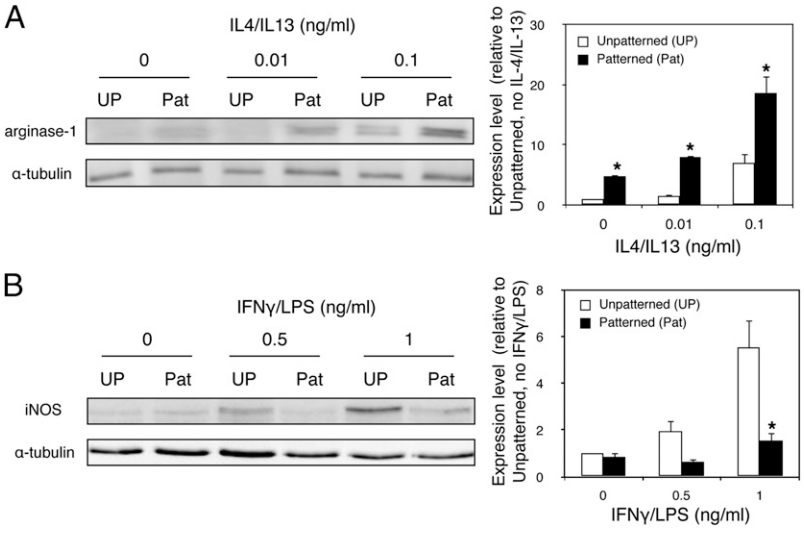

Fig. 4. Shape and cytokines synergize to modulate macrophage polarization. (*A*) Representative Western blot of arginase-1 and α-tubulin in unpatterned (UP) cells and cells patterned (Pat) on 20-μm wide lines treated with the indicated concentration of IL-4 and IL-13 (*Left*) and quantification of average across three separate experiments (*Right*). (*B*) Representative Western blot of iNOS and α-tubulin in unpatterned cells and cells patterned on 20-μm wide lines treated with the indicated concentration of IFN-γ and LPS (*Left*) and quantification of average across three separate experiments (*Right*). Error bars indicate the SEM for three separate experiments. *$P < 0.05$ compared with unpatterned cells treated with the same dose of cytokines as determined by the Student *t* test.

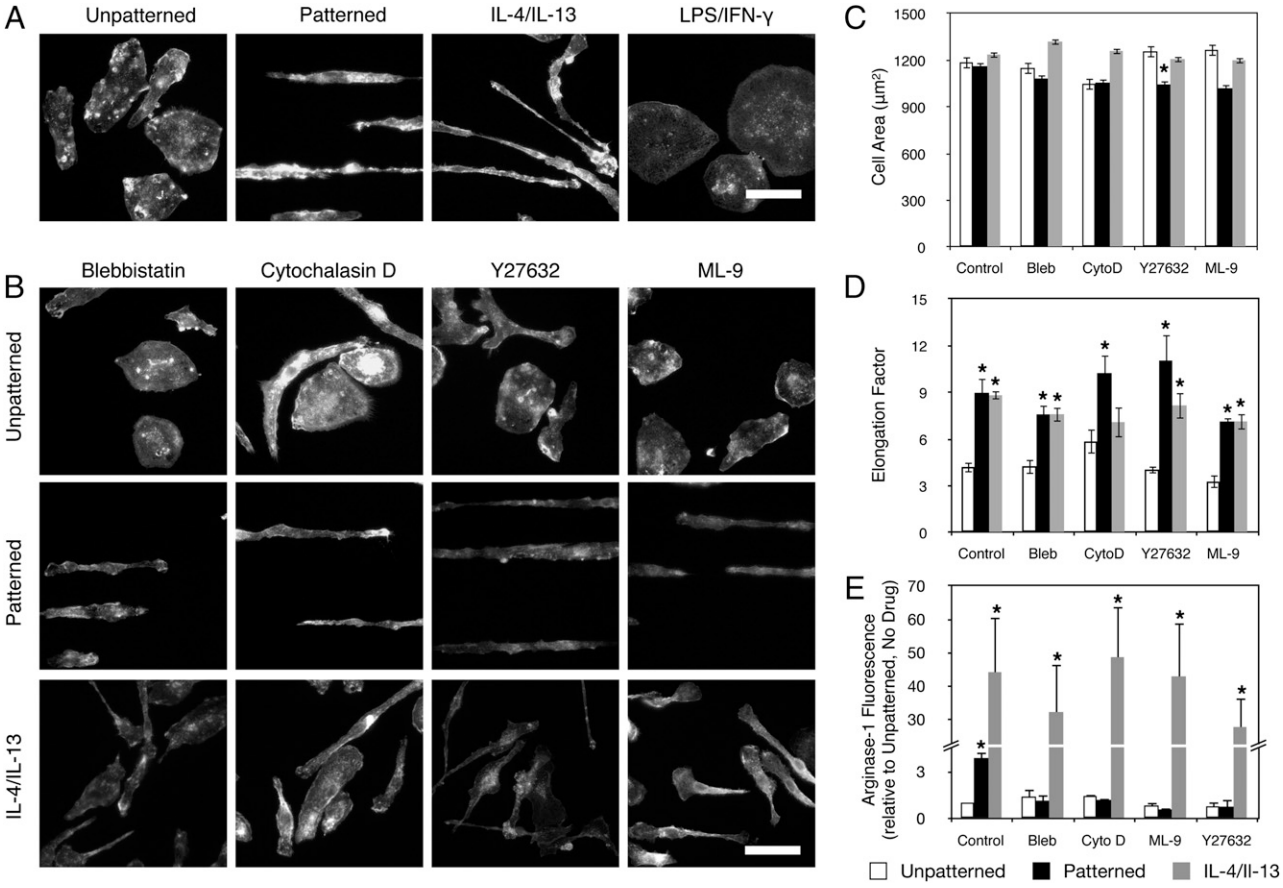

Fig. 5. Cytoskeletal contractility is required for shape-induced M2 polarization. (*A* and *B*) Fluorescence images of phalloidin-labeled unpatterned cells and cells patterned on 20-μm wide lines treated with the indicated pharmacological agents or soluble stimuli. (Scale bar: 50 μm.) Quantification of cell area (*C*) and quantification of cell elongation (*D*) of cells shown in *A* and *B*. (*E*) Relative arginase-1 expression of cells shown in *A* and *B*, as measured by immunofluorescence cytometry. Error bars indicate the SEM for three separate experiments. $*P < 0.05$ compared with unpatterned cells treated with the same drug as determined by the Student *t* test. Bleb, blebbistatin; CytoD, cytochalasin D.

environment associated with collagen remodeling may feed back to regulate arginase-1 expression in macrophages. In addition, our data might help explain why macrophages of different phenotypes can be located in close proximity, where the local tissue architecture may be different but soluble cues are likely to be similar (5, 15). Although the recruitment of distinct precursor populations may contribute to this phenomenon, our data provide compelling evidence that adhesive cues in the local environment can modulate the phenotypic polarization of macrophages.

We demonstrate that contractility within the actin cytoskeleton is a key mediator of shape-induced macrophage polarization. Overall levels of F-actin were higher in M2 and micropatterned cells compared with unpatterned cells, although dramatic changes in cytoskeletal organization were not observed. Although such differences in the cytoskeleton with the macrophage polarization state have been previously reported (21), the functional role of actin in macrophages has been primarily associated with phagocytosis (22), podosome formation (23), or fusion events (24), and little is known about the role of the cytoskeleton in macrophage polarization. We found that abrogation of actin polymerization and actin/myosin contractility, as well as ROCK and MLCK activities, completely eliminated shape-induced effects on polarization, suggesting a critical role for actin in the modulation of macrophage polarization by cell shape. Actin and actin-associated contractility have previously been implicated in transducing shape signals to changes in function in numerous cell types, including endothelial cells, smooth muscle cells, fibroblasts, and stem cells. It is interesting to note that M2 cells, which exhibit elevated actin, have also been shown to demonstrate other characteristics typical of mechanosensitive cells, including a mesenchymal, as opposed to amoeboid, migratory mode (4). However, we found that cytoskeletal contractility was not required for IL-4 and IL-13 to induce arginase-1 expression. Nonetheless, the evidence suggests that although macrophages are derived from a nonadherent monocytic lineage, these cells, particularly those that adopt an M2 phenotype, may indeed be regulated by adhesive interactions with the ECM microenvironment.

How the cytoskeleton transduces physical signals to regulate biochemical cues that modulate the macrophage polarization state remains unknown. Because administration of Y27632 abrogated shape-induced polarization, it is possible that activation of GTPase signaling could participate in pathways that control polarization. In addition, alterations of cell shape may directly influence transcription of M1 or M2 gene programs, because recent evidence has demonstrated a critical role for mechanical signals on transcription factor activity (25). Cellular elongation has been shown to influence nuclear organization, chromatin condensation, and histone modification (10, 26), which could indeed have an impact on genetic programs associated with macrophage phenotype. Furthermore, an important area of future work is to elucidate the effect of elongation on macrophage function.

Dissecting the role of physical cues in macrophage polarization will have broad implications in the treatment of numerous pathological conditions (27). Interestingly, diseases characterized by chronic macrophage activation, including atherosclerosis and cancer, are often associated with alterations in the ECM architecture and mechanics, which are thought to play a critical

role in disease progression (28, 29). Physical properties of the ECM lead to changes in adhesive interactions, integrin and cytoskeletal organization, and thus intracellular signaling pathways that are thought to promote cell transformation (30). A better understanding of how these microenvironmental cues regulate specific disease-associated macrophage functions will be critical for developing immune-targeted therapies for treatment of many diseases. In addition, the ability to modulate immune cell phenotype by the physical environment will be critical for encouraging wound healing in regenerative medicine. The immune response to tissue-engineered scaffold materials is critical to their success, and as such, investigators have explored whether physical factors, such as substrate topology, influence macrophage behavior. Interestingly, it has been observed that surface features indeed affect macrophage morphology and cytokine secretion profiles (31–33), as well as the overall and fibrotic responses (34), although the mechanisms underlying these responses have so far remained elusive. Our data suggest that topological cues might lead to different macrophage responses by influencing their shape and cytoskeleton. Ultimately, encouraging the appropriate immune response to enhance healing will be needed to achieve tissue integration and optimal regeneration.

Materials and Methods

Cell Isolation and Culture. BMDMs were derived from femurs of 6- to 12-wk-old female C57BL/6J mice (Jackson Laboratory) as described in *SI Materials and Methods*. All procedures involving animals were performed in accordance with UC Irvine Institute for Animal Care and Use Committee (IACUC) approved protocols.

Cell Micropatterning and Analysis of Cell Morphology. Cell micropatterning was performed as previously described (17) using stamps with 20-μm and 50-μm wide lines. The long axis, short axis, and cell outline were manually traced from phase contrast images of cells and analyzed for elongation factor and cell area. Details are available in *SI Materials and Methods*.

Immunoassays. Western blotting and flow cytometry were conducted according to standard protocols as described in *SI Materials and Methods* using the following antibodies: goat antiarginase-1 (Santa Cruz Biotechnology), rabbit anti-iNOS (Santa Cruz Biotechnology) or mouse antitubulin (Sigma), HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), FITC rat anti-CD206 (Biolegend), rabbit anti-Ym1 (STEMCELL Technologies), and donkey anti-goat and goat anti-rabbit secondary antibodies (both from Jackson Immunoresearch Laboratories). For cytokine arrays, supernatants were collected and concentrated to 500 μL using 3-kDa centrifugal filters. Concentrated supernatants were tested for cytokines using Mouse Cytokine Antibody Array Panel A (R&D Systems) according to the manufacturer's protocol, and are further described in *SI Materials and Methods*.

Immunofluorescence Imaging and Cytometry. Patterned and unpatterned or polarized BMDMs were introduced to the pharmacological inhibitors blebbistatin (1 μM), cytochalasin D (0.1 μg/mL), Y27632 (50 μM), and ML-9 (0.1 μM) (all from Calbiochem) for 24 h. Immunofluorescence staining was performed as described in *SI Materials and Methods*.

Statistical Analysis. Data were presented as the mean ± SEM, unless otherwise indicated, across at least three independent experiments. For experiments where individual cells were assayed by microscopy, at least 100 cells were examined in each experiment. Comparisons were performed by means of a two-tailed Student t test, and $P < 0.05$ was considered significant.

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