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Differential regulation of macrophage inflammatory activation by fibrin and fibrinogen

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Abstract

Fibrin is a major component of the provisional extracellular matrix formed during tissue repair following injury, and enables cell infiltration and anchoring at the wound site. Macrophages are dynamic regulators of this process, advancing and resolving inflammation in response to cues in their microenvironment. Although much is known about how soluble factors such as cytokines and chemokines regulate macrophage polarization, less is understood about how insoluble and adhesive cues, specifically the blood coagulation matrix fibrin, influence macrophage behavior. In this study, we observed that fibrin and its precursor fibrinogen elicit distinct macrophage functions. Culturing macrophages on fibrin gels fabricated by combining fibrinogen with thrombin stimulated secretion of the anti-inflammatory cytokine, interleukin-10 (IL-10). In contrast, exposure of macrophages to soluble fibrinogen stimulated high levels of inflammatory cytokine tumor necrosis factor alpha (TNF- α). Macrophages maintained their anti-inflammatory behavior when cultured on fibrin gels in the presence of soluble fibrinogen. In addition, adhesion to fibrin matrices inhibited TNF- α production in response to stimulation with LPS and IFN- γ , cytokines known to promote inflammatory macrophage polarization. Our data demonstrate that fibrin exerts a protective effect on macrophages, preventing inflammatory activation by stimuli including fibrinogen, LPS, and IFN- γ . Together, our study suggests that the presentation of fibrin(ogen) may be a key switch in regulating macrophage phenotype behavior, and this feature may provide a valuable immunomodulatory strategy for tissue healing and regeneration.

Keywords

Fibrin; Fibrinogen; Macrophage; Polarization

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Disclosures

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1. Introduction

Fibrin is a fibrous protein involved in blood clotting and is a major component of the provisional extracellular matrix formed after tissue injury. The fibrin network is generated by polymerization of the soluble precursor fibrinogen after its enzymatic cleavage by thrombin, which is activated during tissue damage. The fibrin clot is not only important for homeostasis, but also acts as a scaffold into which platelets, leukocytes and fibroblasts infiltrate and adhere to fill the wound site [1,2]. The provisional matrix is eventually replaced by collagen, fibronectin, and other extracellular matrix (ECM) components to form new tissue [3,4]. Given its natural role in wound healing and the ease of purifying natural fibrinogen from blood, fibrin has been developed and successfully used in the clinic as a tissue sealant [5–7]. It is also currently being explored for applications in tissue engineering and delivery of drugs and growth factors [8–15]. While studies demonstrate that fibrin can be used as a tissue adhesive, how this matrix modulates immune cell function during tissue repair and healing is still not well understood.

Among the leukocytes present during tissue healing, macrophages are considered master regulators, since they are involved in both the initial inflammatory phase as well as the resolution and healing that follow. Macrophages are recruited from circulating monocyte and resident tissue macrophage populations [16,17]. Animals that are depleted of monocytes and/or macrophages exhibit reduced wound healing, suggesting that these cells are essential to the repair process after injury [18,19]. Upon exposure to cytokines released by tissue damage, such as interferon- γ (IFN- γ), or molecules found on pathogenic agents, including lipopolysaccharide (LPS), macrophages adopt a classically activated phenotype and secrete cytokines to promote inflammation. At the later stages of wound healing or upon exposure to Type 2 T helper (Th2) cytokines including interleukin-4 (IL-4) and interleukin-13 (IL-13), macrophages polarize towards an alternatively activated phenotype and facilitate tissue repair by secreting anti-inflammatory cytokines including interleukin-10 (IL-10) and transforming growth factor β (TGF- β) [20]. While chemokines, cytokines and other soluble factors are thought to be the major determinants of macrophage function [21,22], recent evidence suggests that insoluble cues including adhesive geometry, material topography, or stiffness may also influence macrophage behavior [23–28]. As such, changes in the extracellular matrix composition during wound healing may play an active, rather than passive, role in modulating macrophage function. Supporting this idea, it has been shown that the composition of decellularized matrices or extracellular matrix coating on implanted biomaterials influences classical versus alternative activation of macrophages during the host response [29–34]. However, few studies have investigated the role of the provisional matrix, and fibrin in particular, in regulating macrophage function.

Fibrin not only acts as a scaffold into which cells infiltrate but also provides molecular signals to direct cell function, since it contains binding sites for integrins, growth factors and other ECM components including fibronectin. Fibrin and its precursor fibrinogen are thought to interact with Toll-like receptor 4 (TLR-4) and integrins expressed on macrophages including CD11b/CD18 ($\alpha_M\beta_2$, Mac-1, CR3) [35,36] and CD11c/CD18 ($\alpha_X\beta_2$) [37,38]. Fibrinogen is thought to increase inflammatory activation of macrophages largely through binding to TLR4 [39], and upregulation of circulating fibrinogen is often

used as a marker of inflammation in a number of diseases including vascular wall disease, multiple sclerosis, and arthritis [40]. In contrast, evidence also indicates that fibrinogen may promote the alternative activation of macrophages [41]. In the context of biomaterials, the adsorption of fibrinogen to biomaterial surfaces is thought to be important for recruitment of macrophages [42,43]. However, incorporation of fibrinogen into fibrin may alter not only its tethering properties and mechanical presentation but also its protein conformation. Changes in conformation may affect the molecular structure and exposure of binding epitopes [44–48], and thus influence fibrin(ogen)'s interaction with, and downstream signaling in, macrophages. Therefore, it is important to consider the potentially distinct effects of fibrin and fibrinogen on macrophage function.

In this study, we examined the independent and combined effects of fibrin and fibrinogen on macrophage function. We found that culture of macrophages on gels of increasing fibrin content led to a significant increase in the secretion of the potent anti-inflammatory cytokine IL-10, whereas treatment of macrophages with soluble fibrinogen yielded higher levels of TNF- α and relatively lower levels of IL-10, consistent with what has been reported by others [39,49]. Changes in macrophage function and particularly the anti-inflammatory phenotype were not associated with dramatic changes in cell morphology that have previously been observed by our lab and others [23,50]. Interestingly, culture on fibrin completely abrogated TNF- α secretion elicited by fibrinogen or LPS with IFN- γ . Together, these results suggest the presentation of fibrin(ogen) modulates its inflammatory versus anti-inflammatory effects, and that fibrin may be used as part of a strategy to promote anti-inflammatory activity of macrophages.

2. Materials and methods

2.1. Cell isolation and culture

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). Femurs from 6 to 12 week old female C57BL/6J mice (Jackson Laboratory) were harvested. Bone marrow was flushed with Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS (both from Thermo Fisher). Cells were treated with ACK lysis buffer (Thermo Fisher) to lyse red blood cells, centrifuged, and resuspended and cultured in D-10 media. D-10 media is composed of high-glucose DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin (all from Thermo Fisher), and 10% conditioned media from CMG 14–12 cells expressing recombinant mouse macrophage colony stimulating factor (M-CSF) to induce differentiation to bone marrow derived macrophages (BMDMs). After culture for 7 or 8 days, BMDMs were dissociated using cell dissociation buffer (Thermo Fisher) and seeded onto 96 or 24 well tissue culture polystyrene (TCP), round 18 mm diameter No. 1 glass coverslips, or gels fabricated on TCP and glass coverslips in D-10 media. For cytokine or fibrinogen stimulation, macrophages were stimulated six hours post seeding with a combination of 1.0 ng/ml of E. coli-derived LPS (Sigma-Aldrich), 1.0 ng/ml of recombinant murine IFN- γ (R&D Systems), 10 ng/ml of IL-4 (BioLegend), 10 ng/ml IL-13

(BioLegend), or 2.0 and 4.0 mg/ml soluble fibrinogen. Polymyxin B (PMB, InvivoGen) was added at a concentration of 20 µg/ml.

2.2. Gel fabrication

Pure collagen gels containing 2.0 mg/ml protein were fabricated using rat tail Type I collagen (Corning) according to the manufacturer's suggested protocol. Briefly, collagen was mixed with appropriate amounts of 10X PBS (Lonza), 1 N NaOH, and Millipore water. Pure fibrin gels were fabricated at 2.0 mg/ml using bovine plasma Type IS fibrinogen (Sigma), which contains approximately 50 EU/ mg, as measured by ToxinSensor Chromogenic LAL Endotoxin Assay kit (Genescript). The lyophilized protein was reconstituted in PBS containing calcium and magnesium (Sigma). 0.2 U of thrombin was used per milligram of fibrinogen to initiate the polymerization reaction. Collagen-fibrin mixture constructs were formed using rat tail collagen I and bovine fibrinogen in denoted ratios such that the final protein content remained 2 mg/ml. Protein solutions and other reagents were prepared in the same manner as described above. To form 3D gels, TCP or glass coverslips were coated with ECM solutions and incubated in a humidified 37 °C incubator overnight. For studies examining the effects of collagen, fibrin, and fibrinogen, cells were seeded at 50,000 cells/cm². For studies examining the effects of cytokines (LPS, IFN-γ, IL-4, IL-13) in conjunction with fibrin and fibrinogen, or cytoskeletal staining, cells were seeded at 100,000 cells/cm², a density that we have previously used to examine the effect of cytokines on macrophages [51].

2.3. Laser scanning confocal microscopy

For imaging purposes, composite gels were fabricated on 35 mm glass bottom dishes. Laser-scanning confocal back reflection microscopy (backscatter) was performed with an Olympus IX81 microscope. Samples were illuminated with a 488 nm laser light (NTT Electronics Optiλ) with a 40× objective (Olympus). Images were captured using the Olympus Fluoview software. The backscattered light signal was detected with a photomultiplier tube. Fibers differing in refractive indices from their surroundings enabled 3D structure details to be recognized as reflection images were taken along the z-axis at sequential focal lengths.

2.4. Assessment of cytokine secretion

Supernatants of BMDMs cultured on collagen/fibrin composites or fibrin, with and without fibrinogen stimulation, were collected at 36 h after stimulation. Tumor necrosis factor alpha (TNF-α) and interleukin-10 (IL-10) secretion levels were assessed by enzyme-linked immunosorbent assay (ELISA) following manufacturer's protocol (BioLegend). For samples analyzed with a Luminex 32-plex mouse cytokine array and TGF-β Array 3-plex (Eve Technologies), BMDMs were seeded on 2 mg/ml fibrin gels and further stimulated with 4 mg/ml of fibrinogen or external cytokines mentioned in 2.1. Supernatants were collected 4 and 18 h after stimulation. Hierarchical clustering was performed in R utilizing a complete linkage method and presented with the gplots package. Z-scores were calculated for each sample relative to the columnwise mean for presentation.

2.5. RT-PCR

Cells were harvested by scraping for conditions on tissue culture polystyrene; cells on fibrin gels were extracted from the gels with a mortar and pestle. Cells were pelleted and stored at -80°C until RNA was extracted with the Qiagen RNeasy Mini kit. Reverse transcription was performed with the Qiagen Quantitect Reverse Transcriptase kit, which includes a DNase treatment step before RT and uses random hexamer primers. The resulting cDNAs were diluted tenfold in water before qPCR. qPCR was performed with Bio-Rad Ssofast Evagreen master mix on a Bio-Rad CFX96 instrument using the manufacturer's recommended cycling protocol, i.e. a 30 s incubation at 95°C followed by 40 cycles of 5 s at 95°C and 5 s at 55°C . Samples were run in duplicate. Previously optimized primers are described in the Supplemental Information. Forward and reverse primers were both used at 400 nM except for *Tnfa*, where primer concentration was 200 nM. NTCs for all templates showed no amplification. cDNAs were verified to be free of genomic DNA contamination by the ValidPrime assay [52]. C_q s were called by a threshold method. Results were analyzed in R; transcript abundance was determined relative to *Gapdh* using the 2^{-C_q} method. Data and analysis script are available at <https://github.com/WendyLiuLab/Hsieh2016PCR>.

2.6. Fluorescence staining and imaging

For arginase-1 (Arg-1) and inducible nitric oxide synthase (iNOS) staining, BMDMs were seeded on plain or fibrin gel-coated glass coverslips and stimulated with fibrinogen at 6 h after adhesion. Control BMDMs were stimulated with LPS/IFN- γ , IL-4/IL-13, and LPS/IL-4/IL-13 at concentrations described in 2.1. After 18 h, cells were fixed with 100% cold methanol on ice for 15 min. Samples were blocked with both 5% normal donkey and goat serum in PBS (both from Jackson ImmunoResearch). Samples were then incubated with goat anti-arginase-1 and rabbit anti-iNOS (both from Santa Cruz Biotechnology) primary antibodies, followed by Alexa Fluor-594 donkey anti-goat and Alexa Fluor-488 goat anti-rabbit secondary antibodies (both from Jackson ImmunoResearch), and counterstained with Hoechst 33342 (Thermo Fisher). For detection of the actin cytoskeleton, BMDMs were seeded on plain or fibrin gel-coated glass coverslips and further stimulated with fibrinogen. After 4 or 18 h, cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) and washed with PBS (Lonza), and permeabilized with 0.1% Triton X-100 (Sigma) in PBS. Samples were incubated with Alexa Fluor-488 conjugated phalloidin and counterstained with Hoechst 33342 (both from Thermo Fisher). Images of fixed samples were acquired in epifluorescence with an Olympus IX83 inverted microscope with a 40 \times oil immersion lens and Micro-Manager microscope control software [53].

2.7. Statistical analysis

Data presented are from three independent biological experiments and values are represented as mean \pm SEM unless otherwise indicated. Statistical analysis was performed considering $p < 0.05$ to be statistically significant. Data were analyzed using an one-way ANOVA followed by Tukey's HSD post-hoc test or Student's two-sided t -test assuming equal variance followed by false discovery rate correction for multiple comparisons, as indicated in the figure captions.

3. Results

3.1. Composition of fibrin and collagen gels influences fiber architecture and macrophage cytokine secretion

Composite gels composed of fibrin and collagen have been previously explored as scaffolds for biological functions such as angiogenesis [54]. To begin examining whether the provisional matrix formed during wound healing regulates macrophage behavior, we fabricated hydrogels composed of collagen, fibrin, and composite mixtures thereof. Using back reflection microscopy, we observed distinct fiber architectures in gels of different composition: a pure collagen gel exhibited long, thick, branching fibers, whereas a pure fibrin gel exhibited short, thin and non-branching fibers (Fig. 1A). Gels containing a mixture of fibrin and collagen displayed both thin and thick fibers, with finer fibrils observed in gels with higher fibrin concentration. To determine the effect of matrix composition on macrophage function, BMDMs were seeded onto composite gels and secretion of IL-10 and TNF- α were examined by ELISA after 36 h of culture. We observed that secretion of IL-10 was significantly enhanced when macrophages were cultured on gels containing fibrin, with only 0.2 mg/ml (10% of overall protein content) eliciting a significant increase (Fig. 1B). As fibrin content was further increased, the levels of secreted IL-10 concomitantly increased and reached 400 pg/ml on pure fibrin gels. In contrast, TNF- α levels remained relatively low—below 130 pg/ml—across all gel compositions, although there was a modest increase with added fibrin. Together, these data suggest that the addition of collagen to fibrin gels alters its fibrillar architecture and influences the secretion of cytokines by macrophages.

3.2. Presentation of fibrin(ogen) modulates macrophage inflammatory versus anti-inflammatory cytokine secretion

Previous work has suggested that fibrinogen, the soluble precursor of fibrin, stimulates macrophage inflammatory activation [39,49,55]. To specifically explore the effect of fibrin versus fibrinogen, we cultured BMDMs on fibrin gels or on tissue culture polystyrene (TCP) surfaces, stimulated with soluble fibrinogen 6 h post seeding, and then examined cytokine secretion 36 h after stimulation (Fig. 2A and B). We found that increasing concentrations of soluble fibrinogen significantly enhanced the secretion of TNF- α , confirming observations previously reported by others [39]. As little as 0.02 mg/ml of fibrinogen was sufficient to stimulate moderate TNF- α secretion and higher stimulation concentrations of 0.2–4 mg/ml saturated TNF- α levels at approximately 1000 pg/ml. IL-10 secretion also saturated with 0.2–4 mg/ml fibrinogen stimulation, but reached a more moderate level of 200 pg/ml. In contrast, cells cultured on fibrin gels secreted much higher levels of IL-10 and little TNF- α (Fig. 2B). As with the fibrin-collagen composite gels, increasing the concentration of fibrin led to a corresponding increase in IL-10 secretion. Macrophages exposed to gels with a concentration between 1 and 4 mg/ml of fibrin secreted significantly more IL-10 than cells exposed to 1–4 mg/ml soluble fibrinogen. In contrast, TNF- α remained relatively low and was less than 200 pg/ml across gel conditions, although there was still a moderate increase with increasing fibrin concentration. Together, these results demonstrate that fibrin and fibrinogen activate opposing macrophage functions: fibrin is anti-inflammatory while fibrinogen is inflammatory. These data suggest that the molecular presentation of fibrinogen

—either in the soluble form or incorporated within fibrin networks—may play an important role in modulating macrophage inflammatory activation.

3.3. Adhesion to fibrin inhibits fibrinogen-induced inflammatory activation

In the native wound healing environment, it is likely that macrophages are simultaneously exposed to fibrinogen and fibrin, as the precursor molecule is cleaved and incorporated into the gel network. To examine the combinatorial effect of fibrinogen and fibrin, we seeded macrophages on 2 mg/ml fibrin gels or TCP surfaces, further stimulated with different doses of soluble fibrinogen 6 h after seeding, and then analyzed for cytokines secreted into the supernatant at 36 h post stimulation (Fig. 2C). We found that cells cultured on fibrin matrices maintained high IL-10 secretion levels, independent of the presence of fibrinogen (Fig. 2D). The levels of IL-10 secretion by cells cultured on fibrin and stimulated with fibrinogen were significantly higher, by approximately two-fold, than the levels secreted by macrophages stimulated with fibrinogen alone. Interestingly, culture of macrophages on fibrin completely abrogated the TNF- α activated by soluble fibrinogen (Fig. 2D). These effects were likely to be independent of the presence of endotoxin in the fibrinogen, since the addition of the endotoxin inhibitor polymyxin B did not affect the results (Supplemental Fig. 1). These data suggest that the anti-inflammatory effects of fibrin dominate when macrophages are exposed to both fibrin and fibrinogen.

To more comprehensively assess macrophage cytokine secretion profiles elicited by fibrin and fibrinogen, we performed a multiplex analysis of a panel of 35 secreted proteins. BMDMs were seeded on fibrin gels or 24 well TCP wells, further stimulated with soluble fibrinogen, and supernatants were collected at 4 and 18 h after stimulation. Here, the time points were shortened in order to better capture the temporal dynamics of the secreted proteins. In addition, we compared the cytokine levels of cells cultured on fibrin and/or stimulated with fibrinogen to levels from cells stimulated with LPS/IFN- γ , IL-4/IL-13, and LPS/IL-4/IL-13, molecules known to induce macrophage activation. Hierarchical cluster analysis of the Z-score, the number of standard deviations away from the mean across all conditions, suggested that the cells cultured on fibrin and stimulated with fibrinogen were more similar to cells cultured on fibrin alone as opposed to cells stimulated with fibrinogen alone (Fig. 3A and Supp. Fig. 1). We found that the concentrations of inflammatory cytokines elicited by cells stimulated with fibrinogen were mostly comparable to cells stimulated with LPS/IFN- γ , although there were some cytokines that were more highly elicited by LPS/IFN- γ , including IL-6, IL-12, and MIG. Interestingly, IL-10 and G-CSF levels were higher in cells cultured on fibrin compared to cells stimulated with conditions containing IL-4/IL-13, suggesting that adhesion to a fibrin matrix may in fact promote anti-inflammatory activities more effectively than soluble cytokines. Cluster analysis of the entire cytokine panel suggested that cells stimulated with fibrinogen were more closely related to cells stimulated with LPS, in combination with IL-4/IL-13 or IFN- γ , whereas cells cultured on fibrin were more closely related to cells stimulated with IL-4/IL-13 or control, unstimulated cells.

Further examination of cytokines that were secreted at the highest absolute levels revealed that many inflammatory cytokines shared a similar trend with that of TNF- α , including IL-6,

MCP-1, MIG, MIP-1 α , MIP-1 β , and RANTES (Fig. 3B). These cytokines were induced by fibrinogen and their secretion levels decreased when cells were cultured on a fibrin gel, with or without fibrinogen. A few cytokines including IP-10, MIP-2 and KC were upregulated by fibrinogen and not affected by or only moderately diminished (in the case of MIP-2) in the presence of fibrin. Concordant with IL-10, concentrations of cytokines involved in wound healing, including G-CSF and TGF- β 1, were higher in cells cultured on fibrin when compared to cells stimulated with fibrinogen, although TGF β 1 was moderately reduced with the addition of fibrinogen while G-CSF was not. Unstimulated macrophages had the highest concentration of TGF- β 1, which may suggest that the basal activation state in M-CSF-containing media is polarized towards an alternatively activated phenotype [56]. Many of the cytokines examined were upregulated by 4 h and the levels were maintained at 18 h, although IL-6, RANTES, MCP-1, MIG, and G-CSF did not appear at maximal concentrations until the longer time point. Overall, the majority of the cytokines are consistent with TNF- α and IL-10, and suggest that fibrin promotes anti-inflammatory signaling and inhibits inflammatory signaling induced by fibrinogen. Some inflammatory cytokines were induced by both fibrinogen and fibrin, suggesting the involvement of multiple pathways. However, despite the range of results, the cytokine secretion levels from cells cultured on fibrin and further stimulated with soluble fibrinogen were overall more similar to those cultured on fibrin alone as opposed to those stimulated with fibrinogen alone, suggesting that the effects of adhesion to fibrin matrices dominate the response.

3.4. Fibrin(ogen) modulates expression of markers associated with macrophage polarization

To more broadly examine whether fibrin and fibrinogen lead to changes in macrophage phenotype polarization, we assessed the expression of a panel of gene markers by RT-PCR. Cells were cultured on fibrin or TCP for 6 h, and then soluble stimuli including fibrinogen, LPS, and/or cytokines were added for an additional 4 h. Cluster analysis of the entire panel showed that cells cultured on fibrin, with or without fibrinogen, were more similar to control or LPS/IL-4/IL-13 stimulated cells, whereas cells stimulated with fibrinogen were more similar to cells stimulated with LPS and IFN- γ (Fig. 4A). Expression of *Arg1* was consistent with IL-10 secretion and higher in cells cultured on fibrin, with or without additional fibrinogen, when compared to control or fibrinogen only stimulated cells (Fig. 4B). However, amounts of *Nos2*, *Tnfa*, and *Il10* were all upregulated in cells stimulated with fibrinogen and reduced when cells were cultured on fibrin gels. Notably, *Il10* gene expression was not correlated with secreted cytokine levels, perhaps because of the time point used for evaluation. In addition, expression of some genes including *Retnla*, *Chi3l3*, *Kdm6b*, and *Mrc1* did not appear to be affected by fibrin or fibrinogen (Fig. 4A and Supplemental Fig. 3).

We next examined whether adhesion to fibrin or exposure to soluble fibrinogen induced changes in the protein expression of arginase-1 (Arg-1) and inducible nitric oxide synthase (iNOS), established pro-healing and pro-inflammatory macrophage phenotype markers respectively (Fig. 4C). BMDMs were seeded on 2 mg/ml fibrin gels or glass coverslips, stimulated with 2 mg/ml soluble fibrinogen, and then fixed and stained for Arg-1 and iNOS expression at 18 h after stimulation. Expression levels were compared against control cells

that were polarized by treatment with LPS and IFN γ , IL-4 and IL-13, and LPS with IL-4 and IL-13. As expected, control cells stimulated with LPS/IFN γ expressed iNOS and minimal levels of Arg-1 while those stimulated with IL-4/IL-13 and LPS/IL-4/IL-13 expressed Arg-1 but not iNOS. We found that macrophages seeded on glass and exposed to fibrinogen expressed moderate levels of Arg-1 and high levels of iNOS when compared to the unstimulated condition or cells seeded on fibrin gels, with or without fibrinogen. However, macrophages seeded on fibrin gels, with or without fibrinogen, expressed Arg-1 and little iNOS. Together, these data suggest that adhesion to fibrin promotes Arg-1 and inhibits iNOS induced by fibrinogen.

3.5. Fibrin(ogen) elicits changes in cytoskeletal organization and cell shape

Our previous work suggested that cell shape plays a role in the modulation of macrophage phenotype and that cell elongation is associated with an alternatively activated or anti-inflammatory phenotype [23]. To determine if there was an association between cell shape and changes in macrophage function in the context of fibrin and fibrinogen, we evaluated the actin cytoskeleton by phalloidin staining. BMDMs were seeded on 2 mg/ml fibrin gels or glass coverslips, stimulated with 4 mg/ml soluble fibrinogen, and then fixed and stained at 4 and 18 h after stimulation (Fig. 5). At 4 h post stimulation, macrophages cultured on glass and stimulated with fibrinogen appeared to be the most well spread, with considerable lamellipodia formation, when compared to the unstimulated condition or cells seeded on fibrin gels with or without fibrinogen. Cells seeded on fibrin tended to cluster with each other. At 18 h, macrophages stimulated with fibrinogen exhibited most of their actin towards the periphery of the cells with dramatic filopodial extensions. Macrophages cultured on fibrin appeared to also have more extensions by 18 h and were in some cases quite elongated and well spread. Interestingly, the cells cultured on fibrin and further stimulated with fibrinogen were very round, suggesting that fibrin inhibits filopodial extensions induced by fibrinogen observed on glass or, conversely, that fibrinogen inhibits the protrusions formed on fibrin gels. In all cases where cells were cultured on fibrin, phalloidin staining appeared to be more intense when compared to cells cultured on glass and stimulated with fibrinogen.

3.6. Fibrin protects macrophages from inflammatory activation by LPS and IFN- γ

Given that macrophages are also likely to be exposed to cytokines in the native wound healing environment, we also studied the combined effect of fibrin(ogen) and soluble stimuli known to activate inflammatory or anti-inflammatory pathways. Macrophages were cultured on fibrin gels or TCP surfaces, stimulated with fibrinogen, with or without further stimulation with LPS/IFN- γ , IL-4/IL-13, or a combination of LPS with IL-4/IL-13, and analyzed for TNF- α and IL-10 production levels at 36 h after stimulation (Fig. 6). As expected, macrophages seeded on TCP surfaces and stimulated with LPS/IFN- γ or LPS/IL-4/IL-13 had higher levels of TNF- α and IL-10 when compared to unstimulated cells, with the highest level of TNF- α observed with LPS/IFN- γ stimulation and highest level of IL-10 observed with LPS/IL-4/IL-13 stimulation. Consistent with our previous experiment, fibrinogen stimulated both TNF- α and IL-10 and, in this case, the levels of IL-10 were higher, most likely due to the increased number of cells seeded in this experiment. Interestingly, IL-10 secretion stimulated by fibrinogen was inhibited by the co-addition of LPS/IFN- γ , whereas TNF- α level was somewhat enhanced. Neither TNF- α nor IL-10 level

was affected by the co-addition of IL-4/IL-13 or LPS/IL-4/ IL-13. Most strikingly, all conditions in which macrophages were cultured on fibrin exhibited similarly high levels of IL-10 and low levels of TNF- α regardless of further stimulation. The abrogation of TNF- α secretion even in conditions containing LPS/IFN- γ suggests that fibrin is able to exert a protective effect on macrophage inflammatory activation.

4. Discussion

Fibrin is the dominant extracellular matrix component during wound healing after tissue damage or injury but its effect on macrophage function has not been clearly elucidated. We demonstrated that the addition of fibrin into collagen matrices reduced fibril size and enhanced the secretion of the anti-inflammatory cytokine IL-10 by macrophages. Culturing cells on pure fibrin gels or treating them with soluble fibrinogen revealed that the presentation of fibrin(ogen), either immobilized within a fibrin network or as a soluble molecule, modulates macrophage inflammatory versus anti-inflammatory function. Stimulating macrophages with fibrinogen led to inflammatory activation, as indicated by secretion of TNF- α and other inflammatory cytokines including IL-6, MCP-1, MIG, MIP-1 α , MIP-1 β , and RANTES, as well as expression of *Tnfa* and *Nos2* genes associated with inflammatory activation. In contrast, culture of macrophages on fibrin stimulated secretion of IL-10, G-CSF, and TGF- β 1 and expression of *Arg1*, which are associated with inflammation resolution and tissue repair [57]. These results were not likely due to the presence of endotoxin, since culture with the endotoxin inhibitor polymyxin B showed the same effects of fibrin and fibrinogen on TNF- α and IL-10 secretion by macrophages. The switch between inflammatory and anti-inflammatory behavior through the presentation of a single molecule may suggest a simple yet potentially powerful approach to modulating macrophage function.

Our results suggest that culture of macrophages on fibrin elicits a protective effect, preventing inflammatory activation by fibrinogen as well as LPS and IFN- γ , known inducers of classical macrophage activation. Many of the inflammatory cytokines that were released after treatment with fibrinogen, including TNF- α , IL-6, MCP-1, MIG, MIP-1 α , MIP-1 β , and RANTES, were inhibited when macrophages were cultured on fibrin. Similarly, we observed that TNF- α secretion elicited by treatment with LPS/IFN- γ or LPS/IL-4/ IL-13 was also inhibited when macrophages were cultured on fibrin. Since fibrin networks typically form at sites of tissue damage where inflammation is presumably high, it is possible that the matrix itself plays a key role in dampening local inflammation in order to promote the wound healing processes that follow. Interestingly, the level of IL-10 secreted by macrophages cultured on fibrin far exceeded the level produced by macrophages stimulated with LPS/IL-4/IL-13, established stimuli of anti-inflammatory function. These data suggest that signals from the extracellular matrix may in fact be more potent than cytokines that are recognized as regulators of macrophage function.

Fibrinogen is thought to activate macrophage inflammatory signaling primarily through its interaction with Toll like receptor 4 (TLR4) [39], but it also binds to CD11b/CD18 ($\alpha_M\beta_2$ integrin) as well as CD11c/CD18 ($\alpha_X\beta_2$ integrin) and CD51/CD61 ($\alpha_V\beta_3$) [58]. Interestingly, CD11b has been shown to negatively regulate TLR4 signaling, since

macrophages lacking CD11b have an enhanced response to LPS, a major TLR4 agonist [59]. In addition, adsorbed fibrinogen inhibits TLR4 signaling through its interaction with CD11b [41]. It is possible that incorporation of fibrinogen into fibrin or adsorption of fibrinogen onto TCP may expose otherwise cryptic CD11b-binding sites that enhance this inhibitory pathway [48,60]. Moreover, mechanical tethering of fibrinogen either by adsorption or polymerization in fibrin may modulate its binding to TLR4 and/or CD11b. Supporting this idea, stimulation of macrophages with mechanically dissociated fibrin clots yielded an inflammatory response [61]. Further work will be necessary to explore how bimolecular and mechanical context regulates the differential binding interactions between macrophages and fibrin(ogen) and whether changes in receptor binding modulate downstream pathways that regulate inflammatory versus anti-inflammatory signaling.

Fibrin and fibrinogen are not only involved in hemostasis and wound healing, but are also present in many diseases including cardiovascular disease, cancer, and neuroinflammatory diseases. Macrophages are thought to contribute to all of these diseases and our data suggest that the balance between fibrin and fibrinogen may be important in regulating their function during disease progression. In cancer, fibrinogen is thought to play a role in metastasis, since lack of fibrinogen has been shown to diminish tumor metastasis [62,63]. Fibrin is found within tumor tissues [64] and may prevent macrophage inflammatory activation to promote tumor survival. In cardiovascular disease, increased plasma levels of fibrinogen and fibrin degradation products are associated with disease progression [65–68], and these molecules, in addition to fibrin, are found within atherosclerotic lesions but not in normal healthy vessels [69]. However, fibrinogen may have some protective effect because deletion of fibrinogen exacerbates atherosclerosis in low-density lipoprotein receptor and apolipoprotein B-deficient mice [70]. In neuroinflammatory diseases including Alzheimer's disease and vascular dementia, fibrinogen is thought to act as an initial trigger, stimulating innate immune responses that lead to axonal damage [71], and accumulation of fibrin promotes inflammation and prevents tissue repair after neurovascular damage [72–74]. Yet, fibrinogen has also been implicated as a regulator of TGF- β mediated signaling within the central nervous system tissues after vascular damage [75]. Unraveling the molecular pathways that control the switch between anti-inflammatory and inflammatory response of macrophages to fibrin(ogen), as well as their degradation products, will be important for improving our understanding of their role in these important diseases.

The ability to control macrophage inflammatory versus anti-inflammatory behavior by altering the presentation of fibrin(ogen) may be leveraged for the design of materials used for tissue regeneration. Most biomaterials elicit a significant host immune reaction and fibrosis after implantation, although a few recent studies suggest that modulating the immune response and reduction of fibrosis can be achieved [76,77]. However, it still remains unclear how much inflammatory versus anti-inflammatory activation of macrophages is necessary to achieve the optimal host response, since both functions are needed for proper wound healing. It is likely that a tightly controlled dynamic activation pattern will be required. Since fibrinogen and fibrin appear to shift macrophage activation from pro- to anti-inflammatory, engineering biomaterials that utilize this activation switch may help to develop a better understanding of the foreign body response. Furthermore, designing new

materials that dynamically present fibrinogen or fibrin may ultimately help control the response to minimize fibrosis and promote proper tissue regeneration.

5. Conclusions

We demonstrated that fibrin and fibrinogen elicit distinct macrophage responses: culture on a fibrin gels led to secretion of anti-inflammatory cytokines whereas treatment with soluble fibrinogen led to inflammatory cytokine secretion. In addition, fibrin exerted a protective effect on macrophages, preventing inflammatory cytokine secretion induced by fibrinogen as well as LPS and IFN- γ . Together our results show that the presentation of a single molecule, either in the soluble form or tethered within an insoluble matrix, plays an important role in the regulation of macrophage phenotype. These findings contribute to our understanding of how the extracellular matrix regulates macrophages during wound healing and disease and may offer a new strategy for design of biomaterials that can precisely control the host response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2016.09.024>.

Statement of Significance

Fibrin is a fibrous protein resulting from blood clotting and provides a provisional matrix into which cells migrate and to which they adhere during wound healing. Macrophages play an important role in this process, and are needed for both advancing and resolving inflammation. We demonstrate that culture of macrophages on fibrin matrices exerts an anti-inflammatory effect, whereas the soluble precursor fibrinogen stimulates inflammatory activation. Moreover, culture on fibrin completely abrogates inflammatory signaling caused by fibrinogen or known inflammatory stimuli including LPS and IFN- γ . Together, these studies show that the presentation of fibrin(ogen) is important for regulating a switch between macrophage pro- and anti-inflammatory behavior.

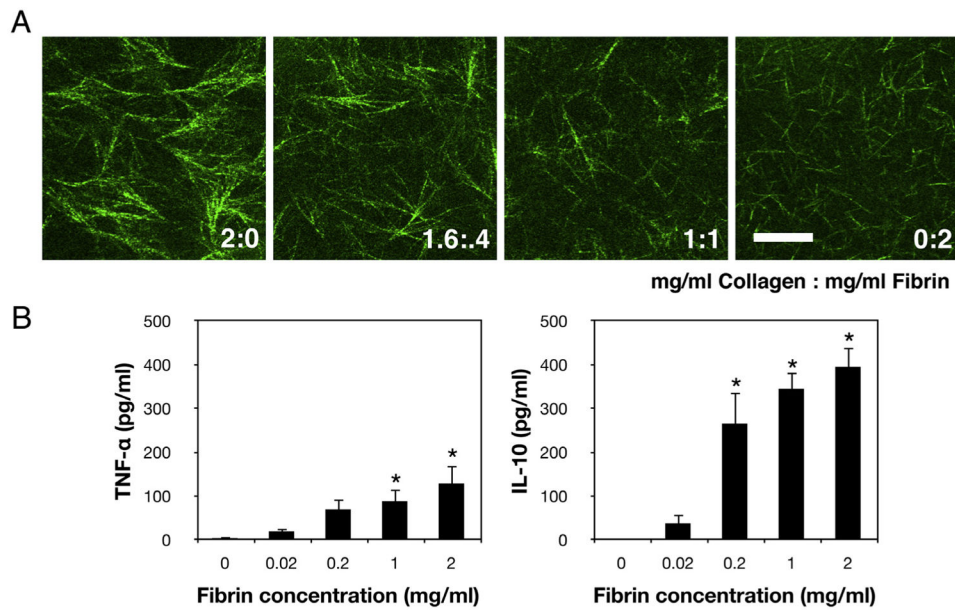
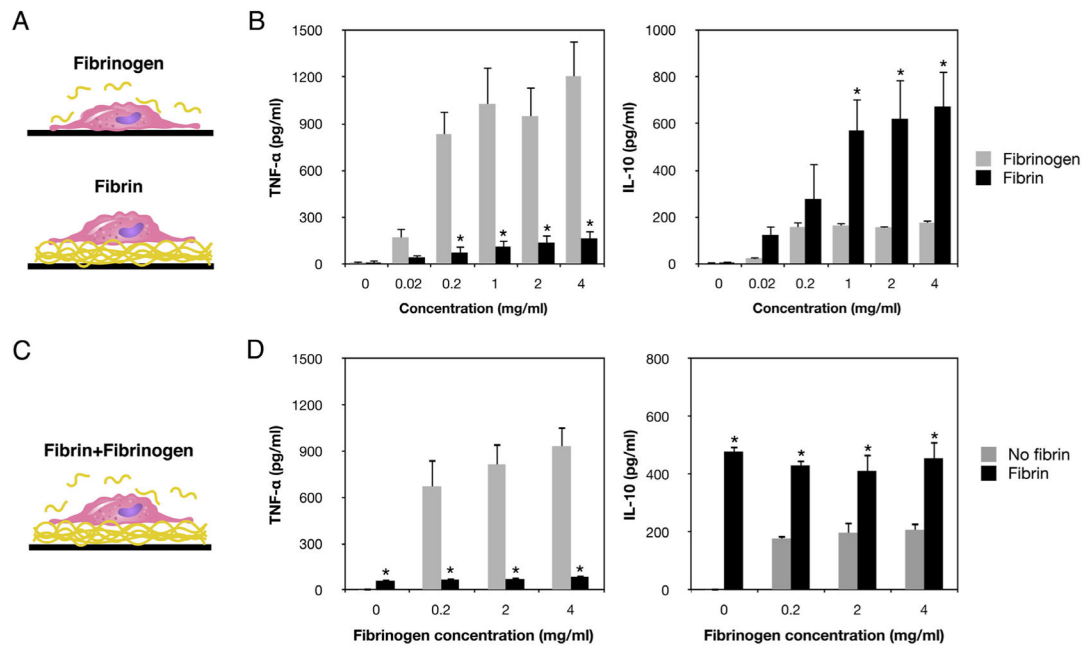


Fig. 1. Composition of collagen-fibrin gels affects fiber architecture and macrophage cytokine secretion. (A) Representative backscatter images of composite collagen-fibrin gels with indicated mg/ml collagen: mg/ml fibrin concentrations. Scale bar: 25 μ m. (B) Graph of TNF- α (left) and IL-10 (right) secretion by macrophages cultured on 2 mg/ml collagen:fibrin composite gels with indicated fibrin concentrations. Values are mean \pm SEM of $n = 3$ biological replicates and asterisks denote $p < 0.05$ by one way ANOVA followed by Tukey's HSD when comparing with the 0 mg/ml fibrin condition.

**Fig. 2.**

Presentation of fibrin(ogen) modulates macrophage cytokine secretion. (A) Schematic of experimental conditions examining the effect of fibrin and fibrinogen on macrophage activation. (B) Graph of TNF- α (left) and IL-10 (right) secretion by macrophages cultured on different concentrations of fibrin or stimulated with different doses of soluble fibrinogen for 36 h. Values are mean \pm SEM of $n = 3$ biological replicates and asterisks denote $p < 0.05$ by Student's t -test followed by false discovery rate correction for multiple comparisons for comparisons within each concentration group. (C) Schematic of experimental conditions to examine the combined effect of fibrin and fibrinogen on macrophage activation. (D) Graph of TNF- α (left) and IL-10 (right) secretion by macrophages cultured on 2 mg/ml fibrin or TCP surface, and stimulated with different doses of soluble fibrinogen for 36 h. Values are mean \pm SEM of $n = 3$ biological replicates and asterisks denote $p < 0.05$ by Student's t -test followed by false discovery rate correction for multiple comparisons for comparisons within each concentration group.

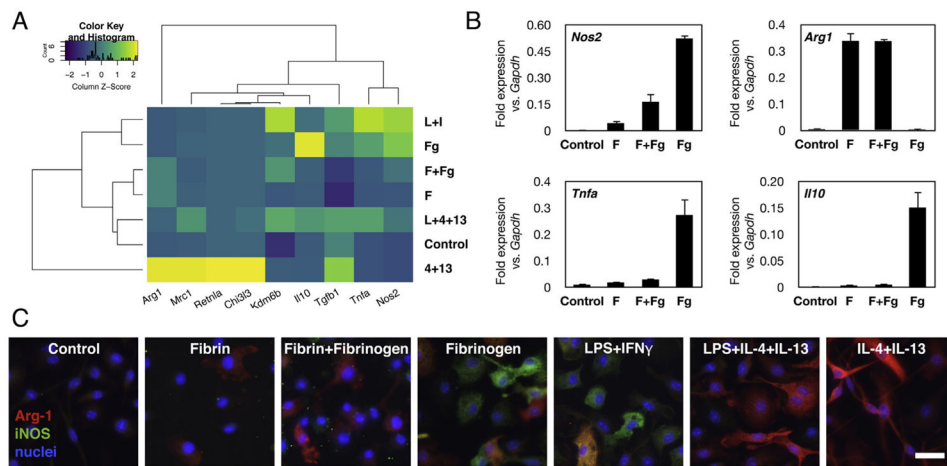


Fig. 4. Fibrin and fibrinogen differentially regulate markers associated with macrophage polarization. (A) Column-wise Z-score normalized heat map of mean gene expression by macrophages after culture on 2 mg/ml fibrin gels with or without 2 mg/ml fibrinogen, or cultured on TCP and stimulated with 2 mg/ml fibrinogen, 1 ng/ml LPS/ IFN- γ (L + I), 10 ng/ml IL-4/IL-13 (4 + 13), or 1 ng/ml of LPS and 10 ng/ml IL-4/IL-13 (L + 4 + 13) for 4 h. (B) Graphs of expression levels relative to *Gapdh* of selected gene expressed by macrophages cultured on 2 mg/ml fibrin with and without 2 mg/ml soluble fibrinogen (F and F + Fg) or cultured on TCP with and without 2 mg/ml soluble fibrinogen (Control and Fg) for 4 h. See Supplemental Materials for information on all genes and conditions shown in A and statistical comparisons. (C) Representative fluorescence images of macrophages cultured in the indicated conditions for 18 h and stained for arginase-1 (red), iNOS (green) or nuclei (blue). Scale bar: 25 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

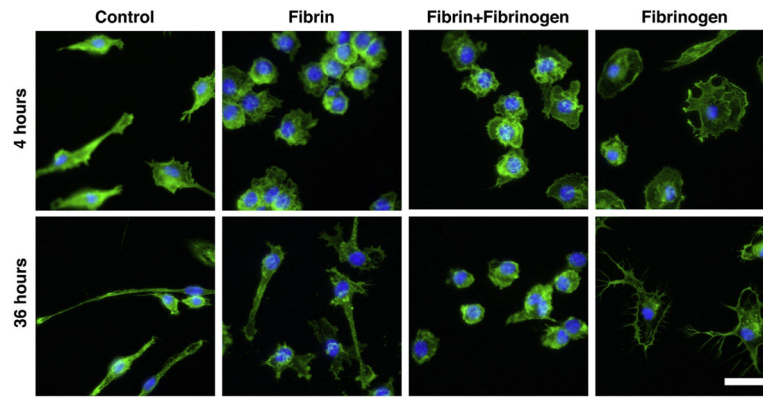


Fig. 5. Actin distribution and cell shape are modulated by fibrin(ogen). Representative fluorescent images of phalloidin (green) and Hoechst (blue)-stained macrophages cultured on glass or 2 mg/ml fibrin and stimulated with 4 mg/ml fibrinogen for 4 or 18 h. Scale bar: 25 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

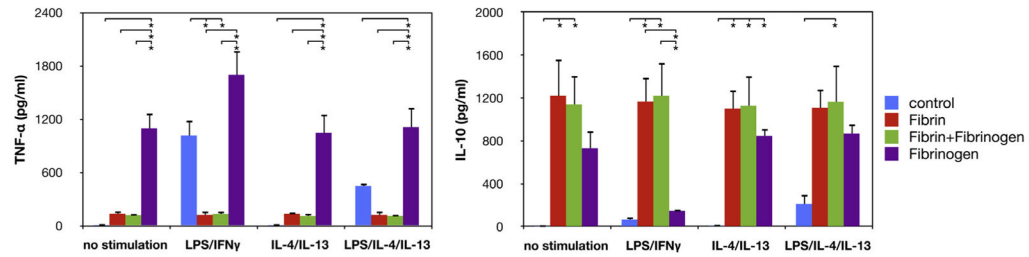


Fig. 6. Effect of fibrin and fibrinogen on macrophage activation by LPS and cytokines. Graph of TNF- α (left) and IL-10 (right) secretion of macrophages seeded on TCP surface or 2 mg/ml fibrin gels, with and without 2 mg/ml fibrinogen, and further stimulated with 1 ng/ml LPS/IFN- γ , 10 ng/ml IL-4/IL-13 or 1 ng/ml of LPS and 10 ng/ml IL-4/IL-13 (LPS/IL-4/IL-13). Values are mean \pm SEM of $n = 3$ biological replicates; asterisk groups denote $p < 0.05$ by one way ANOVA followed by Tukey's HSD test for comparisons within each stimulation group.