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Fibrinogen Stimulates Macrophage Chemokine Secretion Through Toll-Like Receptor 4

Stephen T. Smiley,* Jennifer A. King, † and Wayne W. Hancock‡‡

Extravascular fibrin deposition is an early and persistent hallmark of inflammatory responses. Fibrin is generated from plasma-derived fibrinogen, which escapes the vasculature in response to endothelial cell retraction at sites of inflammation. Our ongoing efforts to define the physiologic functions of extravasated fibrin(ogen) have led to the discovery, reported here, that fibrinogen stimulates macrophage chemokine secretion. Differential mRNA expression analysis and RNase protection assays revealed that macrophage inflammatory protein-1α (MIP-1α), MIP-1β, MIP-2, and monocyte chemotactrant protein-1 are fibrinogen inducible in the RAW264.7 mouse macrophage-like cell line, and ELISA confirmed that both RAW264.7 cells and primary murine thioglycolate-elicited peritoneal macrophages up-regulate the secretion of monocyte chemotactrant protein-1 >100-fold upon exposure to fibrinogen. Human U937 and THP-1 precursor-1 (THP-1) monocytic cell lines also secreted chemokines in response to fibrinogen, upon activation with IFN-γ and differentiation with vitamin D₃, respectively. LPS contamination could not account for our observations, as fibrinogen-induced chemokine secretion was sensitive to heat denaturation and was unaffected by the pharmacologic LPS antagonist polymyxin B. Nevertheless, fibrinogen- and LPS-induced chemokine secretion both apparently required expression of functional Toll-like receptor 4, as each was diminished in macrophages derived from C3H/HeJ mice. Thus, innate responses to fibrinogen and bacterial endotoxin may converge at the evolutionarily conserved Toll-like recognition molecules. Our data suggest that extravascular fibrin(ogen) induces macrophage chemokine expression, thereby promoting immune surveillance at sites of inflammation.


Extravascular fibrin(ogen) may also function by transmitting activating signals to leukocytes. Integrin ligation stimulates “outside-in” signaling (reviewed in Ref. 19), and fibrin(ogen) reportedly promotes CD11b/CD18-dependent NF-κB activation and IL-1β expression by monocytic cells (20, 21). Although relatively high concentrations of fibrin(ogen) are most effective, such levels are probably achieved locally upon fibrin polymerization and deposition. Thus, extravasated fibrin(ogen) may promote both leukocyte adhesion and cytokine secretion at sites of inflammation.

The current study we demonstrate that fibrinogen also stimulates macrophage production of a select set of chemokines that promote attraction of T cells, neutrophils, and additional macrophages. Moreover, we found that fibrinogen- and LPS-stimulated chemokine secretions share a common signaling pathway, each requiring expression of functional Toll-like receptor 4 (TLR4). Thus, evolutionarily conserved Toll proteins apparently signal in response to both foreign pathogens and host fibrinogen. We propose a model in which the relocation of certain host proteins from vascular to extravascular environments may constitute a general means to signal “danger.”

Materials and Methods

Reagents

Cell culture medium, serum, and additives were obtained from Life Technologies (Gaithersburg, MD). Human fibrinogen (certified free of factor XIII, plasminogen, and fibronectin) was purchased from American Diagnostica (Greenwich, CT). Fibrinogen was reconstituted in endotoxin-free water (Sigma, St. Louis, MO), warmed to 37°C for 5 min to complete dissolution, diluted to 2 mg/ml in culture medium, sterile-filtered, and used immediately. Fibrinogen purchased from Sigma and Enzyme Research Laboratories (South Bend, IN) generated similar results. In the experiments reported here, culture medium contained <1 U/ml endotoxin (determined

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3 Abbreviations used in this paper: TLR, Toll-like receptor; PEC, peritoneal exudate cells; MIP-1α, macrophage inflammatory protein-1α; MCP-1, monocyte chemotactrant protein-1.
by Pyrochrome *Limus* Amebocyte Lysate Assay; Associates of Cape Cod, Falmouth, MA), and supplementation with fibrinogen did not significantly increase endotoxin levels. Fibronecin was purchased from Roche (Indianapolis, IN), hirudin from American Diagnostica, human IFN-γ from PharMingen (San Diego, CA), 1α,25-dihydroxyvitamin D₃ from Calbiochem (San Diego, CA), and LPS (Escherichia coli, 0111:B4) and polymyxin B sulfate from Sigma.

**Cells**

Mouse RAW264.7, human U937, and human THP-1 monocytic cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured as recommended by the supplier. Mouse peritoneal exudate cells (PEC) were elicited by i.p. injection of 3 ml 1% thioglycolate broth (Sigma) into 8-wk-old female C57BL/6, C3H/HeJ, and C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, ME). After 72 h, peritoneal cavities were flushed with 7 ml PBS containing 5% FCS and 5 mM EDTA. PEC were then collected by centrifugation and washed with culture medium.

**Cell culture**

Human and mouse cells were cultured in RPMI and DMEM, respectively. All cultures were supplemented with 10% FCS, 2 mM glutamine, 50 μM penicillin, 50 μg/ml streptomycin, 50 μg M-2-ME, and 10 mM HEPES. For some experiments, U937 cells were precultured in 500 μg/ml human IFN-γ, and THP-1 cells were precultured in 50 ng/ml 1α,25-dihydroxyvitamin D₃. Cells were harvested, counted, and seeded at 2 x 10⁵ cells/well in 24-well dishes. Inhibitors and stimuli were added to achieve a final concentration of 1 μM/well. Supernatants were harvested 24 h later for ELISA. In some experiments cells were precultured for 2–16 h to permit adherence, but this did not significantly affect the results.

**Expression arrays and RNase protection assays**

For RNA analyses, RAW264.7 cells were harvested 6 h poststimulation with fibrinogen. RNA was prepared using RNeasy kits (Qiagen, Valencia, CA). Labeled cDNA was prepared, and blots were hybridized as instructed by the Atlas Mouse cDNA Expression Array Kit (CLONTECH Laboratories, Palo Alto, CA). The same RNA was also analyzed by mCK-5 RNase protection assay (PharMingen) as directed by the manufacturer.

**ELISA**

We used human and mouse OptEIA Kits (PharMingen) to quantify macrophage inflammatory protein-1α (MIP-1α) and monocyte chemoattractant protein-1 (MCP-1) protein in culture supernatants. Samples were always sufficiently diluted to ensure that values fell within the linear range of the assay. The values reported depict averages of duplicate cultures, each of which was also diluted and assayed in duplicate. SEMs were always within 15% of the reported mean values.

**Results**

RAW264.7 cells up-regulate mRNA encoding MIP-1α, MIP-1β, MCP-1, and MIP-2 upon exposure to fibrinogen

As macrophages express receptors for fibrinogen, and extravascular fibrinogen is frequently associated with inflammatory responses, we examined functional consequences of macrophage exposure to fibrinogen. Prior studies established that fibrinogen can stimulate IL-1β and TNF-α expression by human monocytes (21, 22). We confirmed those findings (data not shown) and sought to identify other fibrinogen-inducible monocyte/macrophage genes. We cultured the murine RAW264.7 macrophage-like cell line with fibrinogen (500 μg/ml) for 6 h, prepared RNA, converted the RNA to cDNA, and hybridized the cDNA to commercially available filters spotted with probes for 600 mouse genes. Compared with the medium control (Fig. 1A), only one fibrinogen-up-regulated gene was detected: the chemokine MIP-1β (Fig. 1B). Using RNase protection assays, we confirmed MIP-1β up-regulation and also identified three other fibrinogen-inducible chemokine mRNAs: MIP-1α, MIP-2, and MCP-1 (Fig. 1C).

**Fibrinogen stimulates mouse macrophage chemokine secretion**

We next evaluated whether fibrinogen-stimulated increases in chemokine mRNA levels led to corresponding increases in protein secretion. We harvested supernatants from RAW264.7 cells cultured in the presence or the absence of fibrinogen for 24 h and assayed chemokine secretion by ELISA. MCP-1 levels increased 15- and 100-fold above baseline upon exposure of RAW264.7 cells to 50 and 500 μg/ml fibrinogen, respectively (Fig. 2A). Statistically significant (3- to 5-fold) increases were also evident in cultures supplemented with as little as 5 μg/ml fibrinogen. Thus, fibrinogen stimulates RAW264.7 cells to secrete MCP-1 in a dose-dependent manner.

We then assessed whether primary mouse macrophages also secrete chemokines in response to fibrinogen. We injected C57BL/6 mice with thioglycolate and harvested PEC after 72 h, a time at which >70% of the harvested cells are macrophages (data not shown). In a dose-response relationship similar to that seen with the RAW264.7 cells, thioglycolate-elicited PEC also secreted MCP-1 upon exposure to fibrinogen (Fig. 2B). Again, 500 μg/ml fibrinogen stimulated MCP-1 secretion >100-fold. PEC and RAW264.7 cells also secreted MIP-1α protein in response to fibrinogen (data not shown).

**Fibrinogen stimulates chemokine secretion from activated or differentiated human monocytes cells**

Having established that murine macrophages secrete chemokines in response to fibrinogen, we sought to extend our findings to human cells. Initial studies revealed that freshly harvested human PBMC as well as THP-1 and U937 monocytic cell lines failed to significantly up-regulate MCP-1 secretion in response to fibrinogen. However, when we preactivated U937 with 500 U/ml IFN-γ for 24 h, fibrinogen then stimulated >20-fold increases in MCP-1 secretion.
production (Fig. 3A). Unlike U937 cells, THP-1 cells did not acquire fibrinogen responsiveness when pretreated with IFN-γ. However, when THP-1 cells were cultured with vitamin D₃, which promotes differentiation to a more macrophage-like phenotype (23), they then became fibrinogen responsive (Fig. 3B). The failure of fibrinogen to up-regulate MCP-1 production from unprimed/undifferentiated U937/THP-1 cells suggests that fibrinogen-induced chemokine secretion may predominantly be a characteristic of activated macrophages. Accordingly, resting vascular monocytes may be unresponsive to circulating fibrinogen, while activated monocytes and tissue macrophages may promptly secrete chemokines in response to extravasated fibrin(ogen).

**Macrophage responses to fibrinogen are specific and do not require conversion of fibrinogen to fibrin.**

We next evaluated the specificity of fibrinogen-induced chemokine secretion. Adhesion alone was clearly insufficient to stimulate chemokine secretion, as the cells in our control and fibrinogen-stimulated cultures were both adherent to plastic culture dishes. Fibrinogen is a ligand for the macrophage-expressed integrins CD11b/CD18, CD11c/CD18, and CD51/CD61 (α₅β₁) (8–16, 24). To evaluate whether all ligands for macrophage integrins stimulate MCP-1 secretion, we cultured RAW264.7 cells with fibronec tin, another CD51/CD61 ligand (24). In contrast to fibrinogen, fibronec tin did not stimulate macrophage MCP-1 secretion (Fig. 4). Thus, integrin-mediated adhesion is not sufficient to activate chemokine secretion.

Upon proteolysis by thrombin, fibrinogen polymerizes, generating fibrin, an insoluble extracellular matrix. When fibrinogen was added to murine macrophage cultures, low levels of thrombin in culture medium prompted its gradual conversion to fibrin, readily observable as a translucent insoluble gel. To distinguish

**FIGURE 2.** Fibrinogen stimulates the secretion of MCP-1 protein by mouse macrophages. A. RAW264.7 cells (2 × 10⁵) were seeded in 24-well plates and cultured in the presence of the indicated quantities of fibrinogen. After 24 h, supernatants were collected, and secreted MCP-1 protein was quantified by ELISA. B. C57BL/6 mice were injected with 3 ml thioglycolate, and PEC were harvested 72 h later. PEC were then assayed for MCP-1 secretion by ELISA. For both A and B, similar results were observed in at least six independent experiments.

**FIGURE 3.** Human monocytic cell lines respond to fibrinogen upon activation and differentiation. A. Human U937 cells were precultured in the presence or the absence of 10 ng/ml human IFN-γ for 24 h before assay for MCP-1 secretion by ELISA. B. Human THP-1 cells were precultured in the presence or the absence of 50 ng/ml vitamin D₃ for 72 h before assay for MCP-1 secretion by ELISA. Each experiment was repeated twice.

**FIGURE 4.** Macrophage responses to fibrinogen are specific and do not require conversion of fibrinogen to fibrin. Thioglycolate-elicited PEC from C57BL/6 mice were assayed for MCP-1 secretion in response to 50 μg/ml fibrinogen, 50 μg/ml fibrinogen supplemented with 1 U/ml hirudin, or 50 μg/ml fibronec tin. After 24 h, supernatants were harvested and assayed for MCP-1 by ELISA. Similar results were obtained with RAW264.7 cells (data not shown) and were replicated in a second experiment.
roles for fibrin and fibrinogen in promoting macrophage chemokine secretion, we supplemented cultures with hirudin, a pharmacologic thrombin antagonist (25). Hirudin (1 U/ml) suppressed all detectable endogenous thrombin activity, as assessed both by microscopic examination for fibrin formation and by a sensitive quantitative thrombin assay (Spectrozyme TH; American Diagnostica; data not shown). However, fibrinogen-induced MCP-1 secretion was unaffected by the presence of hirudin (Fig. 4). Thus, fibrinogen-stimulated chemokine secretion does not require the conversion of fibrinogen to fibrin.

**Fibrinogen-stimulated macrophage chemokine secretion is LPS independent**

LPS is a potent activator of macrophage activities. Indeed, like fibrinogen, LPS up-regulates macrophage expression of mRNA encoding MIP-1α, MIP-1β, MIP-2, and MCP-1 (data not shown) and secretion of MCP-1 (Fig. 5). To establish that fibrinogen-induced chemokine secretion could not be explained by LPS contamination, we supplemented cultures with polymyxin B, a well-characterized pharmacologic LPS antagonist. Although polymyxin B did not significantly affect fibrinogen-induced MCP-1 secretion by PEC (Fig. 5A), it clearly suppressed LPS-stimulated MCP-1 secretion (Fig. 5B). We obtained similar results with RAW264.7 cells (Fig. 5, C and D) and IFN-γ-primed U937 cells (data not shown). Polymyxin B also failed to suppress fibrinogen-stimulated increases in MIP-1α, MIP-1β, MIP-2, and MCP-1 mRNA (data not shown). Although published studies routinely use 10 μg/ml polymyxin B to antagonize LPS, polymyxin B did not significantly affect fibrinogen-induced chemokine secretion even at 25 μg/ml; higher concentrations were toxic to macrophage cultures. Control experiments using suboptimal doses of fibrinogen supplemented with stimulating doses of LPS established that fibrinogen does not prevent polymyxin B from inactivating contaminating LPS (data not shown).

To further establish the LPS independence of fibrinogen-induced chemokine secretion, we assayed heat-denatured fibrinogen. Like many proteins, boiling fibrinogen for 5 min significantly reduced its functional activity, evidenced by a dramatic reduction in its capacity to induce MCP-1 secretion from RAW264.7 cells (Fig. 5C). In contrast, boiled LPS remained active (Fig. 5D). Together, the temperature-sensitive nature of the stimuli and the failure of polymyxin B to inhibit the response indicate that fibrinogen-induced macrophage MCP-1 secretion is LPS independent.

**Fibrinogen-stimulated macrophage chemokine secretion is TLR4 dependent**

As fibrinogen and LPS both transmit signals prompting macrophage chemokine secretion, we evaluated whether they function through similar pathways. TLR4 was recently implicated in responses to LPS (26–28). As C3H/HeJ mice express mutant TLR4 (26, 27), we analyzed fibrinogen-induced chemokine production from C3H/HeJ macrophages. In comparison to genetically similar, but TLR4-sufficient, PEC from C3H/HeOuJ mice, fibrinogen failed to stimulate MCP-1 secretion from C3H/HeJ macrophages. Again, this result could not be explained by LPS contamination, as polymyxin B suppressed LPS-induced, but not fibrinogen-induced, MCP-1 secretion by C3H/HeOuJ PEC (Fig. 6). Thus, macrophage responses to both LPS and fibrinogen require functional TLR4.

**Discussion**

Fibrinogen is a 340-kDa multimeric glycoprotein that has critical functions in vascular hemostasis. Although fibrinogen normally circulates in plasma at concentrations approximating 3 mg/ml, its levels can exceed 7 mg/ml during inflammatory responses. At sites of inflammation, endothelial cell retraction permits extravasation

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**FIGURE 5.** Fibrinogen-stimulated chemokine secretion is not LPS dependent. A and B, Thioglycolate-elicited PEC were stimulated with the indicated quantities of fibrinogen or LPS in the presence or the absence of the pharmacologic LPS antagonist polymyxin B sulfate (10 μg/ml). After 24 h supernatants were harvested and assayed for MCP-1 by ELISA. C and D, RAW264.7 cells were similarly assayed after being stimulated with native or boiled fibrinogen (50 μg/ml) or LPS (10 ng/ml) in the presence or the absence of polymyxin B sulfate (10 μg/ml). Each experiment was performed at least twice.
Fibrinogen-stimulated chemokine secretion requires functional TLR4. Thiglycolate-elicited PEC were harvested from C3H/HeOuJ and C3H/HeJ mice, which possess wild-type and mutant Tlr4 genes, respectively. Cells were stimulated with the indicated quantities of fibrinogen (A) or LPS (B) for 24 h in the presence or the absence of polymyxin B sulfate (25 μg/ml) and assayed for MCP-1 secretion by ELISA. Similar results were obtained in three independent experiments.

FIGURE 6. Fibrinogen-stimulated chemokine secretion requires functional TLR4. Thiglycolate-elicited PEC were harvested from C3H/HeOuJ and C3H/HeJ mice, which possess wild-type and mutant Tlr4 genes, respectively. Cells were stimulated with the indicated quantities of fibrinogen (A) or LPS (B) for 24 h in the presence or the absence of polymyxin B sulfate (25 μg/ml) and assayed for MCP-1 secretion by ELISA. Similar results were obtained in three independent experiments.
triggered by pathogens (40, 41), and TLR critical for signaling the presence of LPS have been identified in mice, humans and Drosophila (26–28, 41–43). C3H/HeJ mice express mutant TLR4 and thus respond poorly to LPS (26, 27). We found that C3H/HeJ PEC also respond poorly to fibrinogen (Fig. 6), suggesting that fibrinogen and LPS signaling converge at TLR4. Indeed, all previously identified fibrinogen-inducible genes are also inducible by LPS.

As fibroblasts and endothelial cells can express TLR4 (44–47), we suspect that fibrinogen-stimulated chemokine secretion by those cells is also TLR4 dependent. Likewise, we predict that fibrinogen-induced leukocyte NF-κB activation (20) and expression of TNF-α (22) and IL-1β (21) are TLR4 dependent. Notably, under certain experimental conditions, full leukocyte responses to LPS require the expression of both TLR4 and CD11b/CD18 (48). Thus, associations between TLR4 and CD11b may account for the prior studies suggesting roles for CD11b in some fibrinogen-stimulated responses (20, 21, 29). We hypothesize that integrin-mediated binding may sometimes help to initiate fibrinogen-stimulated responses, but that signal propagation will proceed through TLR4.

Formally, we have yet to demonstrate that fibrinogen signals directly through TLR4. Rather, we found that C3H/HeJ macrophages, which express mutant TLR4, fail to respond to fibrinogen. It is conceivable that the expression of TLR4 may enable fibrinogen responsiveness indirectly, perhaps by affecting the priming or differentiation of macrophages. Indeed, TLR4 apparently regulates aspects of macrophage priming/differentiation, as C3H/HeJ macrophages express altered basal levels of IFN regulatory factors (49). Although future studies will be required to distinguish direct and indirect roles for TLR4 in fibrinogen signaling, our data clearly establish that macrophage chemokine secretion in response to fibrinogen requires the expression of functional TLR4.

Medzhitov and Janeway (50, 51) recently demonstrated striking evolutionary conservation in Toll pathways. Drosophila and mammals not only possess homologous TLR, but also express conserved downstream adapter molecules, kinases, and transcription factors (reviewed in Ref. 52). In Drosophila, TLR function in both host defense and embryonic development. In the developmental pathway, Toll functions downstream of a serine protease cascade that prompts cleavage-induced multimerization of Spatze (reviewed in Ref. 53) (Fig. 7). In the host defense pathway, Spatze and an as yet to be defined protease(s) also function upstream of Toll (54, 55) (Fig. 7). Notably, despite extensive downstream conservation, upstream homologues have yet to be defined in mammalian TLR-dependent responses.

However, there are striking structural similarities between Spatze and coagulogen, a clotting factor of the horseshoe crab Limulus (56, 57). In Limulus, macrophage-like hemocytes respond to LPS by activating a serine protease cascade that prompts cleavage-induced multimerization and gelation of coagulogen (Fig. 7), a reaction that is the basis of the widely used Limulus assay for endotoxin (58–60). Thus, in flies and crabs pathogens trigger serine protease cascades prompting multimerization of structurally homologous targets, Spatze and coagulogen. We note that in mammals, an upstream serine protease cascade prompts cleavage of fibrinogen, resulting in its multimerization and deposition as fibrin. Thus, fibrinogen, Spatze, and coagulogen are functional homologues, each polymerizing upon activation of an upstream serine protease cascade (Fig. 7). These remarkable interspecies homologies suggest that fibrinogen-activated TLR-dependent mammalian responses may have evolved from Spatze-activated Drosophila responses.

To facilitate sterilizing immunity, the host must recognize the presence of danger and then attract and activate effector leukocytes (61, 62). When danger takes the form of infectious microbes, detection may proceed through host receptors that recognize characteristic pathogen-associated molecular patterns. The ensuing response to “non-self” promptly signals the production of chemo- kines and cytokines, stimulating immune cell accumulation and activation, respectively.

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**FIGURE 7.** Evolutionary relationships between upstream mediators of TLR signaling. The top line of the chart depicts a general scheme in which an extracellular serine protease cascade generates a ligand for a transmembrane receptor, thus prompting intracellular signaling and gene transcription. A. A cascade of this nature is critical for Drosophila (Fly) embryogenesis (53). In that pathway, the extracellular serine protease Gastrulation-Degradative (GasD) cleaves and activates the serine protease Snake, which, in turn, cleaves and activates the serine protease Easter. Easter then cleaves Spatze, prompting its multimerization, thereby generating a ligand for the transmembrane-spanning receptor Toll. Stimulation of Toll activates intracellular signaling pathways, releasing Cactus from the transcription factor Dorsal, and initiating gene transcription. B. Spatze, Toll, Cactus, and Dorsal function similarly in mammalian responses. C. In the horseshoe crab Limulus, a homologous serine protease cascade involving factor C, factor B, and proclotting enzyme is activated by bacterial endotoxins (59, 60). The Limulus cascade culminates in the cleavage-induced multimerization of coagulogen, a close structural homologue of Spatze (56, 57). Downstream signaling events in the crab have yet to be described. D. In mice and humans, microbial products trigger TLR activation, releasing the Dorsal homologue NF-κB from the Cactus homologue IκB (50, 52). These events stimulate transcription of inflammation-associated cytokines and chemokines. As we found that fibrinogen stimulates chemokine secretion via TLR, and as a serine protease cascade (factor VII > factor X > thrombin) activates the conversion of fibrinogen to fibrin (2), we suggest that this pathway evolved from the homologous Drosophila and Limulus inflammatory pathways.
Although signaling danger in response to non-self is an attractive means to initiate microbe-specific immunity, pathogens may circumvent this mode of recognition through evolution. Presumably to combat such scenarios, mammals also evolved mechanisms that facilitate indirect recognition of infectious agents: T cells perceive altered self (i.e., self MHC molecules that adopt non-self structures upon binding foreign peptides), NK cells respond to missing self (i.e., altered or down-regulated self MHC molecules), and macrophages and dendritic cells respond to stressed self (i.e., self heat shock proteins released from necrotic cells (63, 64). Notably, responses to heat shock proteins are also TLR4 dependent (65).

Here we propose another reliable means for host cells to detect danger: innate recognition of relocated self (i.e., host proteins that have been relocated in response to tissue damage). Fibrinogen is normally confined to the vasculature, but at sites of inflammation increased vascular permeability allows plasma extravasation. Relocated fibrinogen may then stimulate macrophage secretion of MIP-1α, MIP-1β, MIP-2, and MCP-1, chemokines that attract T cells, neutrophils, and macrophages. As these cells also express fibrinogen-binding integrins, extrapolated fibrinogen probably stimulates both leukocyte recruitment to and retention at sites of inflammation.

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